

Temporally resolved early BMP-driven transcriptional cascade during human amnion specification

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Abstract

Amniogenesis, a process critical for continuation of healthy pregnancy, is triggered in a collection of pluripotent epiblast cells as the human embryo implants. Previous studies have established that BMP signaling is a major driver of this lineage specifying process, but the downstream BMP-dependent transcriptional networks that lead to successful amniogenesis remain to be identified. This is, in part, due to the current lack of a robust and reproducible model system that enables mechanistic investigations exclusively into amniogenesis. Here, we developed an improved model of early amnion specification, using a human pluripotent stem cell-based platform in which the activation of BMP signaling is controlled and synchronous. Uniform amniogenesis is seen within 48 hours after BMP activation, and the resulting cells share transcriptomic characteristics with amnion cells of a gastrulating human embryo. Using detailed time-course transcriptomic analyses, we established a previously uncharacterized BMP-dependent amniotic transcriptional cascade, and identified markers that represent five distinct stages of amnion fate specification; the expression of selected markers was validated in early post-implantation macaque embryos. Moreover, a cohort of factors that could potentially control specific stages of amniogenesis was identified, including the transcription factor TFAP2A. Functionally, we determined that, once amniogenesis is triggered by the BMP pathway, TFAP2A controls the progression of amniogenesis. This work presents a temporally resolved transcriptomic resource for several previously uncharacterized amniogenesis states and demonstrates a critical intermediate role for TFAP2A during amnion fate specification.

eLife assessment

This study presents an **important** dataset that captures the transition from epiblast to amnion using a novel *in vitro* model of human amnion formation. The supporting evidence for the authors' claims is **convincing**. Key strengths of the study include the efficiency and purity of the cell populations produced, a high degree of synchrony in the differentiation process, comprehensive benchmarking with single-cell data and immunocytochemistry from primate embryos, and the identification of critical markers for specific differentiation phases. A notable limitation, however, is the model's exclusion of other embryonic tissues.

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Introduction

The amniotic epithelium is the innermost layer of the amniochorionic membrane that physically divides the maternal and fetal environments (Miki et al., 2005; Miki and Strom, 2006). Amniogenesis is initiated during implantation in humans (and in non-human primates), eventually leading to the formation of an amniotic sac structure that surrounds and protects the developing embryo (Enders et al., 1983; Enders et al., 1986; Miki et al., 2005; Sasaki et al., 2016; Shahbazi and Zernicka-Goetz, 2018; Taniguchi et al., 2019). At implantation, the embryo (referred to at this time as a blastocyst) contains three functionally distinct cell types: a collection of unpolarized pluripotent epiblast cells (precursors to the embryo proper and amniotic ectoderm), a surrounding layer of polarized trophoblast (a placental tissue precursor) and an underlying extraembryonic primitive endoderm (a yolk sac precursor). During implantation, the pluripotent epiblast cells undergo apico-basal polarization to form a cyst with a central lumen, the future amniotic cavity (Carleton et al., 2022). This event is followed by the fate transition of pluripotent epiblast cells at the uterine-proximal pole of the cyst to squamous amniotic ectoderm, forming a sharp boundary between amnion and pluripotent epiblast portions of the cyst (Shahbazi and Zernicka-Goetz, 2018; Shao and Fu, 2022; Taniguchi et al., 2019). This structure, the amniotic sac, represents the substrate for the next essential steps of embryonic development. Recent transcriptomic studies of early human and non-human primate embryos have presented benchmark resources for the amnion transcriptome of primates (Bergmann et al., 2022; Nakamura et al., 2016; Nakamura et al., 2017; Sasaki et al., 2016; Tyser et al., 2021; Yang et al., 2021).

The mechanistic dissection of human amnion development presents a challenge, since it is ethically unacceptable to manipulate human embryos *in vivo* and technically difficult to carry out such studies *in vitro*. Non-human primate (NHP) embryos such as rhesus macaque are a suitable alternative to human embryos, due to their close ancestral similarities (Nakamura et al., 2021), and have already provided crucial insights into molecular processes that could potentially regulate amniogenesis. Indeed, genetic manipulation has been demonstrated in NHP systems to study peri-implantation embryogenesis (Yang et al., 2021), although this is not yet a widely applicable approach due to resource availability and logistical complexity of such studies, which also carry their own unique ethical challenges.

Previously, we developed an *in vitro* system of human amnion development called Gel-3D (Shao et al., 2017a; Shao et al., 2017b), in which human pluripotent stem cells (hPSC) are plated on a soft gel substrate consisting of extracellular matrix (ECM). Within 24 hours, aggregates of hPSC form polarized cysts composed of columnar pluripotent cells. Over the next three to four days, a

3D environment is introduced by supplementing the culture medium with a diluted ECM overlay. Under these conditions, the initially pluripotent cysts spontaneously undergo squamous morphogenesis, lose pluripotency markers, and begin to express amnion markers; in over 90% of such cysts, the central lumen becomes entirely surrounded by squamous amnion cells (hPSC-amnion, (Shao et al., 2017a [DOI](#))). Using this system, we showed that mechanically activated Bone Morphogenetic Protein (BMP) signaling can trigger amniogenesis and identified several genes that are now used as amnion markers (e.g., GATA3, TFAP2A, GABRP, (Shao et al., 2017a [DOI](#); Shao et al., 2017b [DOI](#))). These findings were supported by later studies using early human embryo-like models as well as by single cell transcriptomic studies in early human and NHP embryos (Chen et al., 2021 [DOI](#); Tyser et al., 2021 [DOI](#); Yang et al., 2021 [DOI](#); Zheng et al., 2019 [DOI](#); Zheng et al., 2022 [DOI](#))).

Though this Gel-3D system (Shao et al., 2017a [DOI](#)) has been valuable in allowing initial investigations of the mechanisms underlying amniogenic differentiation, it suffers from the fact that the initiation of amniogenesis by mechanical cues is stochastic and is not fully penetrant, hindering reproducible examinations into temporally and spatially sensitive processes. To better implement mechanistic studies, we developed a highly reproducible hPSC-based amniogenic system, called Glass-3D^{+BMP}, that does not rely on the mechanically activated spontaneous BMP signaling to initiate amniogenesis. Rather, in Glass-3D^{+BMP}, BMP4 ligand is added exogenously, and the amniotic differentiation response is quickly seen in all cells. Interestingly, while BMP signaling activation is immediately detectable in all cells, squamous morphogenesis (starting approximately 24 hours after BMP4 addition) is initially focal within a given cyst, but then spreads laterally, to form fully squamous amnion cysts by 48 hours. Such amnion cysts (hPSC-amnion) are uniformly composed of mature amniotic cells that share similar transcriptomes with amnion cells from a Carnegie stage (CS) 7 human embryo (dataset from (Tyser et al., 2021 [DOI](#))).

Given the robust and synchronous nature of this system, we perform detailed time-course bulk RNA sequencing analyses of developing Glass-3D^{+BMP} hPSC-amnion to comprehensively characterize changes in transcriptional characteristics during the initial stages of amnion fate specification. Strikingly, from 0 to 48 hours after BMP treatment, developing hPSC-amnion displays transcriptomic characteristics of epiblast, intermediate or amnion cells that are present in the CS7 human embryo, revealing a potential amnion differentiation trajectory. Moreover, we systematically characterize dynamically expressed genes, and identify immediate-, early-, intermediate- and late-responding genes associated with amniotic differentiation, some of which are validated and characterized in detail in NHP embryos. Interestingly, in contrast to the previous idea that TFAP2A is a *pan*-amnion marker expressed throughout amniogenesis, we establish that TFAP2A is an early/intermediate responding gene, with a temporal pattern of activation that is distinct from immediate responding genes such as GATA3, another *pan*-amnion marker. Functional analyses show that, in the absence of TFAP2A, amniogenesis is incomplete, suggesting that TFAP2A controls amnion fate progression. Lastly, we identify a population of cells at the boundary of the amnion and the epiblast in the peri-gastrula macaque embryo that shares striking molecular and cellular characteristics of cells undergoing amnion specification in Glass-3D^{+BMP}. Together, using the Glass-3D^{+BMP} amnion platform, these studies present a transcriptomic map of the early stages of amnion differentiation, and identify a critical transcriptional pathway dependent on TFAP2A, as well as a potential site of active amniogenesis in the primate peri-gastrula.

Results

Generation of fully squamous hPSC-derived amniotic cysts in a reproducible and controlled manner using a Glass-3D^{+BMP} culture system

Targeted mechanistic investigations into amnion fate specification has been limited by the fact that amniogenesis is not fully penetrant in existing *in vitro* embryo-like models (Chen et al., 2021 [↗](#); Nasr Esfahani et al., 2019 [↗](#); Shao et al., 2017a [↗](#); Shao et al., 2017b [↗](#); Zheng et al., 2019 [↗](#)), prompting us to explore alternative strategies. Since our previous studies have established that BMP signaling is a major driver of amniogenesis (Shao et al., 2017a [↗](#); Shao et al., 2017b [↗](#)), we sought to establish a system in which we could directly control the activation of BMP signaling. We began with a culture condition called Glass-3D, in which aggregates of hPSC are cultured in a stiff glass substrate condition with the culture medium supplemented with a 3D ECM overlay (Fig. 1A [↗](#)). Under these conditions, aggregates of hPSC initiate radial organization and form cysts with a central lumen within 48 hours (Shao et al., 2017a [↗](#); Taniguchi et al., 2017 [↗](#); Taniguchi et al., 2015 [↗](#)). Importantly, these cysts are composed entirely of polarized pluripotent cells.

When we examined the polarized membrane spatial proteomics of these hPSC-pluripotent cysts, we found that BMP receptors are enriched at the basolateral membrane territory (Wang et al., 2021 [↗](#)). To test whether BMP signaling could be directly activated in these cysts by the addition of BMP to the medium, pluripotent cysts (derived from H9 (WA09) human embryonic stem cells for all experiments, unless otherwise noted) were treated with exogenously provided BMP4 at d2 (Fig. 1B [↗](#), Movie 1, Glass-3D^{+BMP}). Nuclear localization of phosphorylated SMAD1/5 (pSMAD1/5), as detected by immunofluorescence (IF), was used as a readout for BMP signaling activation (Fig. 1C [↗](#)). At d2 in Glass-3D, prior to the addition of BMP, a polarized pluripotent (SOX2⁺) cyst structure has formed, and a small central lumen is visible (Fig. 1C [↗](#) – 0-min); this structure is similar to that of the developing epiblast immediately prior to the initiation of amniogenesis (Shahbazi and Zernicka-Goetz, 2018 [↗](#); Taniguchi et al., 2019 [↗](#)). Strikingly, within 10 minutes after BMP addition, uniform nuclear pSMAD1/5 enrichment is seen in all treated cells at an equivalent level (Fig. 1C [↗](#) – 10-min, fluorescent quantitation in Fig. 1D [↗](#)). This is quickly followed by the expression of GATA3, a *pan*-amnion marker and a well-known immediate downstream BMP target that has been shown to facilitate pluripotency exit (Gunne-Braden et al., 2020 [↗](#)). GATA3 expression is detected in all cells within 3 hours (Fig. 1E [↗](#)), suggesting that BMP signaling is uniformly activated. Interestingly, TFAP2A, another transcription factor thought to be an early *pan*-amnion marker, is not detectable in GATA3 positive cells 3 hours after BMP addition (Fig. 1E [↗](#)). After 48-hr of BMP4 treatment, all cysts show uniform squamous morphogenesis, loss of pluripotency markers (NANOG, SOX2) and activated expression of additional *pan*-amnion markers, TFAP2A and ISL1 (Fig. 1F-H [↗](#)).

To investigate whether exogenous BMP4 ligand is continuously needed throughout the 48-hour time-course in order to form fully squamous hPSC-amnion, d2 pluripotent cysts were cultured with BMP4 for 24 hours (d2 to d3), then cultured without BMP4 for the following 24 hours (d3 to d4), and then harvested at d4 (4 days after initial plating, Fig. 1B [↗](#)). Interestingly, these 24-hr BMP treatment samples show fully squamous amnion cysts that are morphologically and molecularly similar to those treated over 48-hr. That is, they show uniformly squamous cyst morphogenesis and prominent expression of GATA3, TFAP2A and ISL1, (Fig. 1I,J [↗](#), quantitation for fluorescent intensity and nuclear aspect ratios in Fig. 1K,L [↗](#)), as well as relatively similar nuclear pSMAD1/5 signals (Fig. 1M [↗](#)). Moreover, in the 24-hr BMP treatment samples (Fig. 2A [↗](#)), time-course analyses show that heterogeneous GATA3 expression is visible in a few nuclei at 3-hr and 6-hr,

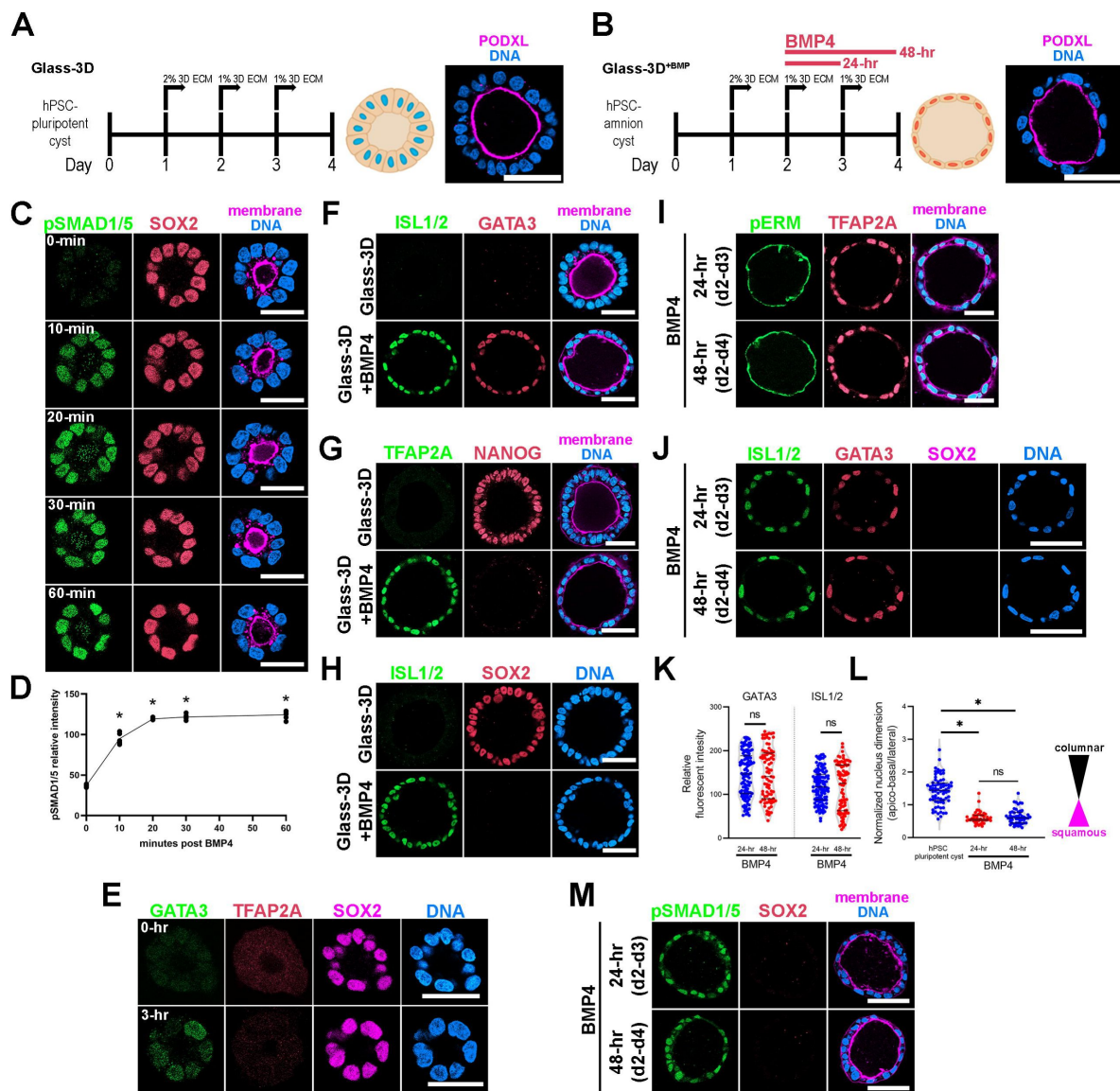


Figure 1.

Highly penetrant amniogenesis in a Glass-3D^{+BMP} culture system.

(A,B) hPSC-derived pluripotent cyst (hPSC-pluripotent cyst, Glass-3D in (A)) and amnion cyst (Glass-3D^{+BMP} in (B)) assays: IF images show hPSC-pluripotent (A) and -amnion (B) cysts stained for PODXL (lumen, magenta) and DNA (nuclear shape, blue). BMP4 treatment (20ng/mL) leads to uniformly squamous amnion cysts by Day 4. (C) Confocal optical sections of d2 cysts harvested prior to (0-min) and 10-, 20-, 30- and 60-min after BMP4 treatment, stained with indicated markers. Staining for phosphorylated SMAD1/5 reveals a prominent nuclear pSMAD1/5 enrichment within 10-min of BMP4 treatment in all cells. (D) Quantification of nuclear pSMAD1/5: five independent samples were counted, more than 50 cells were counted per sample. * indicates statistically significant changes ($p < 0.05$) compared to 0-min timepoint. (E) Abundant GATA3 expression (in green), but not TFAP2A (red), is seen by 3 hours after BMP4 treatment while SOX2 expression is maintained. (F-H) Pluripotency (NANOG, SOX2) and amnion (ISL1/2, GATA3, TFAP2A) marker expression analyses of d4 cysts grown in the Glass-3D (top) or in Glass-3D^{+BMP} (bottom) conditions. (I,J) Confocal micrographs of d4 cysts grown in the Glass-3D^{+BMP} system stained for phosphorylated EZRIN, RADIXIN and MOESIN (pERM), an apical membrane marker, as well as with indicated amnion markers (top – cysts were treated with BMP4 for 24 hours between d2 and d3; bottom – treated with BMP4 for 48 hours from d2 to d4). (K,L) Quantification of GATA3 and ISL1/2 fluorescent intensities in 24- (blue, $n = 115$ cells) and 48-hr (red, $n = 76$ cells) BMP4 treatment samples (K), as well as of nuclear aspect ratios of d4 hPSC-cyst (black, $n = 63$ cells), and 24- (blue, $n = 45$ cells) and 48-hr (red, $n = 52$) BMP4-treated cysts (harvested at d4), (L). (M) pSMAD1/5 staining (green) shows similar nuclear enrichment between 24- and 48-hr BMP4 treatment at d4 (also stained with indicated markers). Scale bars = 50 μ m.

becoming more prominent by 12-24-hr (**Fig. 2B-D**). Early GATA3 expression is followed by initiation of TFAP2A expression by 12-hr (**Fig. 2B,D**). Two other “pan-amnion” genes, *ISL1* and *HAND1* become activated by 24-hr (**Fig. 2C,E**), revealing a previously unrecognized GATA3 → TFAP2A → ISL1/HAND1 amniotic gene activation order (**Fig. 2B,C**, mRNA expression levels in **Fig. 2D,E**).

Importantly, SOX2 expression is abundant at 6-hr, fades in some nuclei at 12-hr and is largely diminished by 24-hr (**Fig. 2B,C**, mRNA expression levels in **Fig. 2F**). NANOG expression follows a similar time course to that of SOX2, revealing a gradual loss of pluripotency (**Fig. 2F**). Samples treated with BMP4 for 24-hr or 48-hr display similar levels of amnion and pluripotency markers (**Fig. 2G**), suggesting that BMP4 treatment may be dispensable after the first 24 hours. To test this possibility, we treated cysts with BMP4 for 24 hours (d2-d3) and subsequently treated with LDN-193189, a small molecule inhibitor for the BMP receptor kinase activity (d3-d4). This treatment resulted in fully squamous cysts that are positive for GATA3 and ISL1/2 (**Fig. 2H**), confirming that BMP signaling initiates the early amniogenesis cascade, but is not required for its progression.

Together, these results show that BMP4 treatment of d2 pluripotent cysts in a Glass-3D culture environment provides a robust amniogenic model that enables mechanistic investigations. We confirmed this finding in a human induced pluripotent stem cell line (1196a, **Fig. S1A,B**, (Chen et al., 2014)) as well as in H7 human ESC (**Fig. S1C**), further establishing this protocol as an effective model of amnion fate conversion *in vitro*. Given that BMP4 treatment from d3 to d4 is dispensable, 24-hour BMP4 treatment between d2 and d3 is used in all remaining experiments using Glass-3D^{+BMP}, unless otherwise noted.

Transcriptomic characterization of developing Glass-3D^{+BMP} hPSC-amnion

To comprehensively understand the transcriptomic changes during hPSC-amnion development in the Glass-3D^{+BMP} system, we performed bulk RNA sequencing analyses for samples taken at 0-, 0.5-, 1-, 3-, 6-, 12-, 24- and 48-hr timepoints (post-BMP4 treatment). Samples were also taken from pluripotent monolayers and d4 pluripotent cysts (**Fig. 3A** (experimental outline), **3B** (heatmap), **3C** (principal component analysis (PCA)), see **Table 1A** for normalized and filtered counts). Overall, a total of 12,891 genes were detected among all sequenced samples. Similar to known immediate BMP-responding genes (e.g., *ID2*, *BAMBI*), *GATA3* shows a significant increase by 1-hr, and continuously increases overtime (**Fig. 3D**, >100-fold increase by 1-hr). Other markers also display expected expression patterns (**Fig. 3E-G**). For example, *TFAP2A* and *ISL1* show significant increases by 3-hr and 24-hr, respectively (**Fig. 3E**), and pluripotency markers (**Fig. 3G**, *NANOG*, *OCT4/POU5F1*, *SOX2*) show reduction overtime. A comparison of gene expression between d4 Glass-3D pluripotent cysts and d4 Glass-3D^{+BMP} hPSC-amnion (48-hr timepoint) shows 1,820 upregulated and 2,092 down-regulated (cut-off = 2-fold, p<0.05) genes in d4 Glass-3D^{+BMP} hPSC-amnion (**Fig. 3H**, see **Table 1B,C** for full lists). Highly upregulated markers include known advanced amnion state markers such as *GABRP*, *IGFBP3* and *EPAS1* (**Fig. 3H**), and highly downregulated genes include *SOX2* and *NANOG* (**Fig. 3H**, see **Table 1D,E** for Gene Ontology (GO) enrichment analyses (Biological Processes) for all significantly upregulated and downregulated genes (> 2-fold, FDR < 0.05)).

The transcriptomic characteristics of developing amnion cells from a Carnegie stage (CS) 7 human embryo were recently described by Tyser *et al.* (**Fig. 4A** shows a UMAP (Uniform Manifold Approximation and Projection) plot with the original coordinates and annotations, (Tyser et al., 2021)). In this analysis, the authors state that yolk sac and connecting stalk tissues were discarded during dissection, but amnion tissue was intact and included in the single cell preparation that was analyzed (Tyser et al., 2021). Consistent with this notion, our analysis in which expression was superimposed on the UMAP plot shows that pan-amnion markers such as

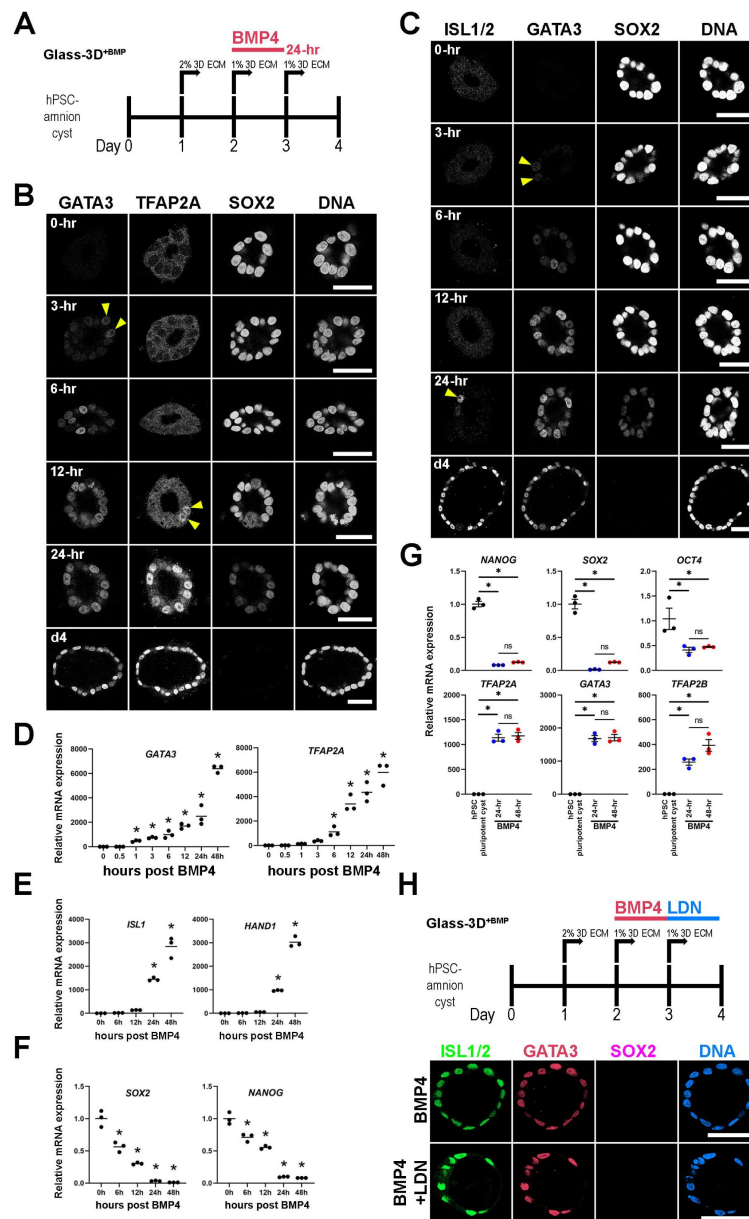


Figure 2.

Time-course analysis of Glass-3D^{BMP} hPSC-amnion development.

(A) Timeline of the Glass-3D^{BMP} hPSC-amnion assay. Twenty four-hr BMP treatment is sufficient to generate uniformly squamous hPSC-amnion cysts. (B,C) Optical sections of time-course Glass-3D^{BMP} hPSC-amnion cysts (harvested at indicated timepoints after BMP4 treatment) stained with indicated markers (inverted black and white fluorescent signals shown for individual channels, to aid visualization). GATA3, an immediate BMP target is seen by 3-hr (yellow arrowheads). hPSC-amnion cysts with prominent nuclear TFAP2A (B) and ISL1/2 (C) are seen by 12- and 24-hr, respectively (yellow arrowheads), while SOX2 shows gradual decrease. Consistent changes are also observed at mRNA transcript levels using quantitative RT-PCR (D-F, also see the expression of additional markers: *HAND1* (amniotic) and *NANOG* (pluripotent); *indicates statistically significant changes ($p < 0.05$) compared to 0-hr timepoint). (G) Relative mRNA expression analyses of indicated pluripotency (top) and amniotic (bottom) markers between d4 Glass-3D hPSC-pluripotent cyst (black), and d4 Glass-3D^{BMP} hPSC-amnion with 24-hr (blue) or 48-hr (red) BMP treatment; * indicates statistically significant changes ($p < 0.05$). (H) BMP inhibitor (LDN-193189) treatment outline as well as confocal micrographs of d4 Glass-3D^{BMP} hPSC-amnion (top, control – 24-hr BMP4 treatment between d2 and d3) and hPSC-amnion treated with LDN-193189 between d3 and d4 after BMP4 treatment (between d2-d3). Scale bars = 50 μ m.

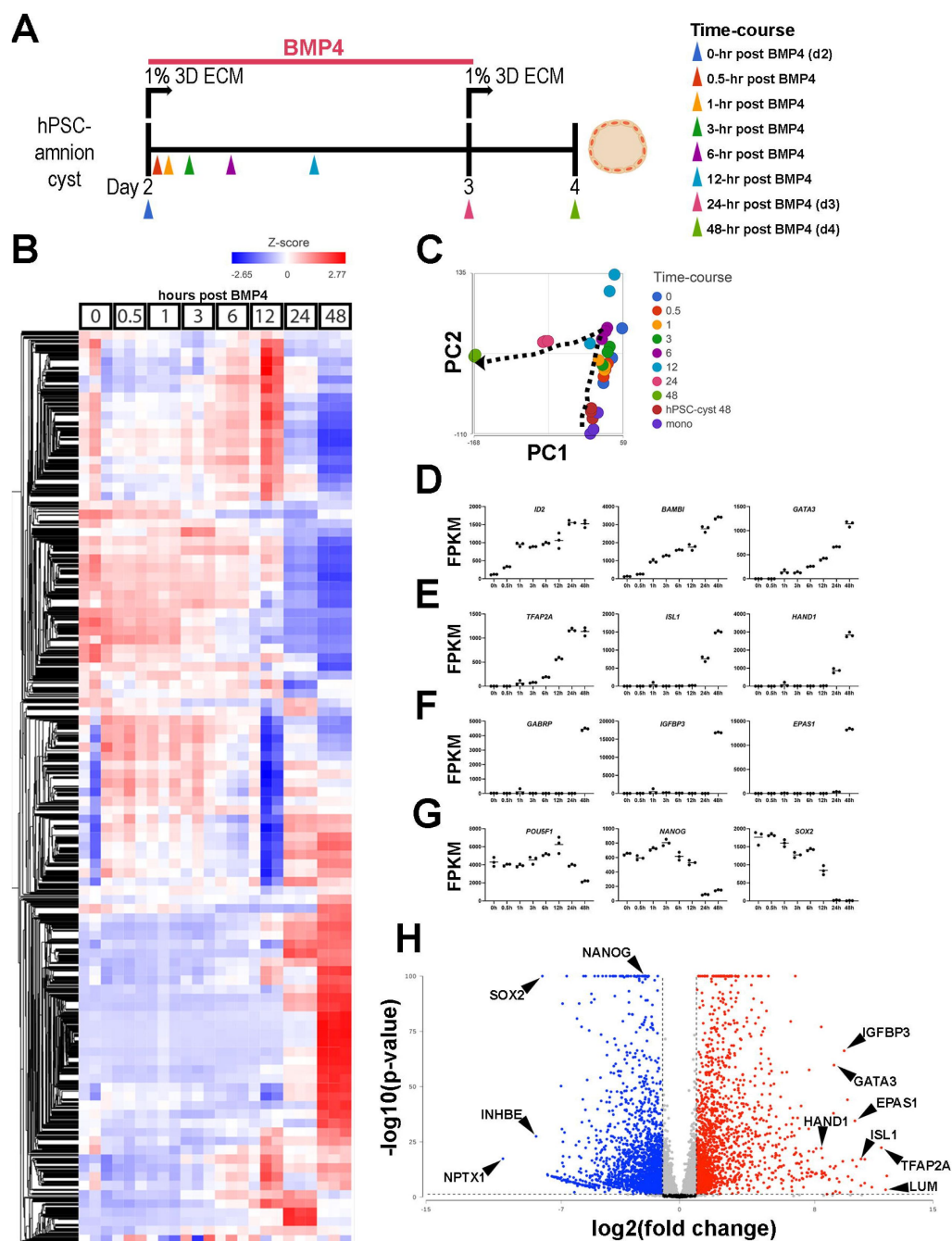


Figure 3.

Temporally-resolved transcriptomic characterization of Glass-3D^{BMP} hPSC-amnion.

(A) Experimental timeline (from d2) of the time-course bulk RNA sequencing analysis. Colored arrowheads indicate timepoints at which samples were harvested. **(B)** A heatmap showing a hierarchical clustering analysis of all detected genes (12,940): bulk transcriptomes from triplicate samples are shown at each timepoint. A gradient scale indicates Z-score. **(C)** Principal component analysis (PCA) plot of time-course bulk transcriptomes, using the most variable genes, revealing progressive transcriptomic changes overtime (indicated by the dotted arrow). Note that coloring is consistent with the arrowheads in **(A)**. **(D-G)** Time-course plots of normalized expression values for selected known immediate BMP target **(D)**, general amnion **(E)**, advanced amnion **(F)** and pluripotency genes **(G)**. **(H)** A volcano plot analysis showing up-(red) and down-(blue) regulated genes in Glass-3D^{BMP} hPSC-amnion cysts (gene expression compared to d4 pluripotent hPSC-cysts): black lines represent cutoffs for p-value <0.05 and 2-fold difference.

GATA3, *TFAP2A* and *ISL1* are broadly expressed in the rod-shaped cell population marked “Ectoderm” (Fig. 4B, areas within the inset “i” are shown in 4B-D; see Fig. S2A for uncropped UMAP expression plots), suggesting that this region contains cells of the amniotic lineage. We next examined which cells in the CS7 human embryo share transcriptomic similarities to developing hPSC-amnion, and found that, indeed, the 48-hr Glass-3D^{+BMP} samples exhibit clear transcriptional similarities to cells marked “Ectoderm” in the CS7 human embryo (Fig. 4C), validating the human amnion-like characteristics of our hPSC-amnion model. Interestingly, the earlier timepoints also show overlaps with cell types of the CS7 human embryo (Fig. 4C, uncropped plot shown in Fig. S2B). While pluripotent monolayer, d4 Glass-3D pluripotent cyst, as well as 0-, 0.5-, 1-, 3- and 6-hr samples transcriptomically resemble Tyser “Epiblast” cells (orange dots in Fig. 4C), 12-hr samples show transcriptomic similarities with cells labeled in the Tyser analysis as “Primitive Streak”; these cells map close to the Epiblast/Primitive Streak boundary in the Tyser UMAP (green dots in Fig. 4C). By 24-hr, the transcriptomes are most similar to Tyser “Primitive Streak” cells proximal to the amniotic “Ectoderm” (blue dots in Fig. 4C). Despite their “Primitive Streak” annotation, these 24-hr cells adjacent to the amniotic “Ectoderm” population display relatively low levels of *TBXT* (a major marker of primitive streak cells, Fig. 4D, (Beddington and Robertson, 1999; Tyser et al., 2021; Zheng et al., 2019)), marking them as distinct from the *TBXT*^{high} “Primitive Streak” cells progressing to “Nascent Mesoderm” (Fig. 4D).

Indeed, in developing hPSC-amnion, while not detectable using IF (Fig. S2C), a *TBXT*^{low} transcriptional state is seen starting at the 24-hr timepoint (Fig. S2D). A similar trajectory as well as gene expression patterns are also seen in the Yang *et al.* (Yang et al., 2021) cynomolgus macaque peri-gastrula single cell RNA sequencing dataset (Fig. 4E-G, uncropped plots shown in Fig. S2E,F). These bioinformatic analyses suggest that the Glass-3D^{+BMP} amnion may develop from pluripotent epiblast-like cells that are transcriptomically equivalent to the CS7 epiblast cells, and, then traverse *TBXT*^{low} intermediate states as the lineage specification continues before acquiring an amniotic fate by 48-hr (dotted arrow, Fig. 4C).

Given that the hPSC-amnion differentiation trajectory in the Glass-3D^{+BMP} model overlaps with cells in the CS7 human embryo, it appears that active amniogenesis processes may be present in the early post-implantation embryo. Thus, we applied an RNA velocity analysis (Bergen et al., 2020) to the Tyser data; in such an analysis, lineage progression is inferred from comparing spliced and unspliced mRNA. Fig. 5A (inset “i”) shows that the Tyser velocity data show differentiation trajectories very similar to the one inferred from the time-course analysis of the Glass-3D^{+BMP} model.

To further validate this notion in a non-human primate system, we performed detailed IF expression analyses for *GATA3*, *TFAP2A*, *ISL1* and *SOX2* in an early cynomolgus macaque embryo staged between CS6 and CS7 (Fig. 5B-E). In this CS6/7 embryo, a clear amniotic sac is seen, with *SOX2*⁺ epiblast cells on one side and contiguous *ISL1*⁺ squamous amniotic ectoderm cells that are also positive for *GATA3* and *TFAP2A* on the other (Fig. 5B-E, fluorescent intensity quantitation shown in 5C,E). Surface ectoderm bridging neural ectoderm and amniotic ectoderm is not seen at this stage; this finding is consistent with the previous histological studies in human (the Carnegie Collection, found in the Virtual Human Embryo Project) and NHP pre- and peri-gastrula embryos (Enders et al., 1983; Enders et al., 1986; Yang et al., 2021).

Although amniotic mesenchyme is not evident, some mesenchymal cells are observed underlying the epiblast (Fig. 5F, green dotted bracket). These mesenchymal cells show abundant nuclear pSMAD1/5 (Fig. 5F) and *TBXT* (Fig. S2G); these cells are likely derived from the primitive streak. Strikingly, at the boundary between the epiblast and amnion, a gradual transition in cell morphology is readily seen: while cells most proximal to the epiblast are columnar, there is a gradual reduction in height until fully squamous cells are seen (Fig. 5B,D,F,H), represented by the changes in nuclear aspect ratio (Fig. 5H). Molecularly, the expression of *GATA3*, *TFAP2A*, *ISL1* and *SOX2* is correlated with such columnar to squamous cell shape change (Fig. 5B-E).

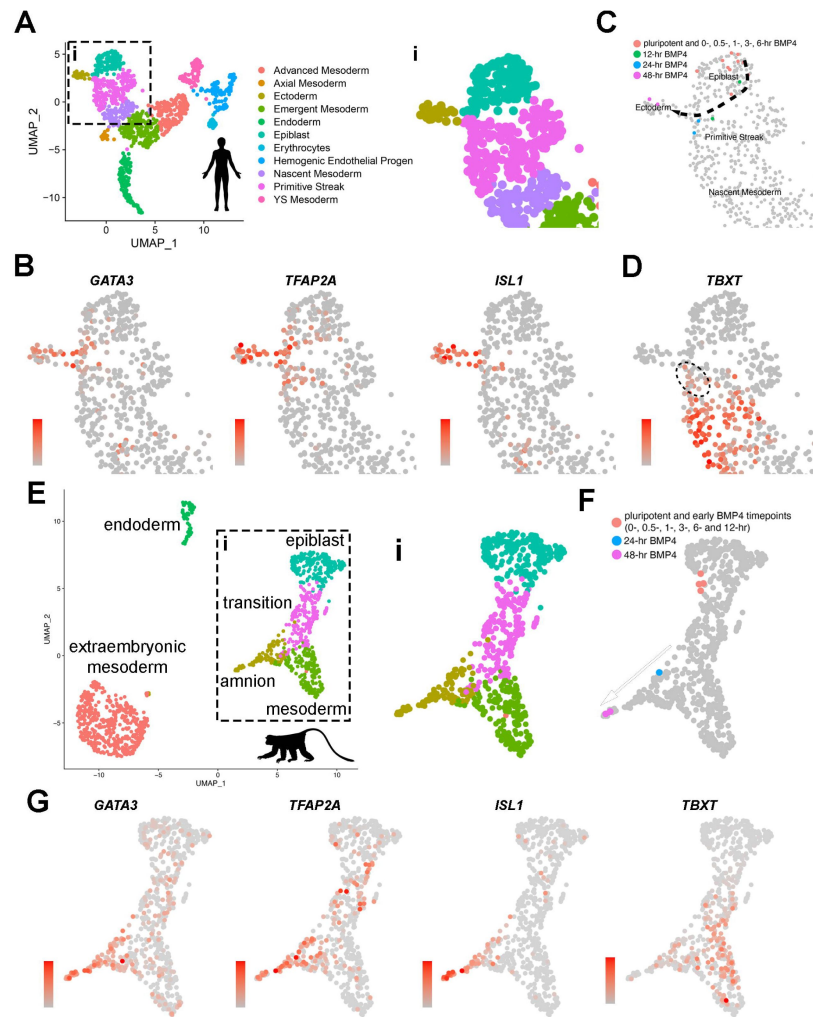


Figure 4.

Transcriptome comparison of developing Glass-3D^{+BMP} hPSC-amnion cysts, a Carnegie stage (CS) 7 human embryo, and two GD14 cynomolgus macaque peri-gastrula.

(A) A UMAP plot displaying the original single cell transcriptome coordinates of the CS7 human embryo described in Tyser *et al.*, shown with the original annotations. The inset (i) is used for expression analyses in (B). (B) Expression of amnion markers – *GATA3*, *TFAP2A* and *ISL1* – superimposed onto the UMAP plot in (A – i). (C) Transcriptome similarity analysis comparing the CS7 human embryo single cell dataset and the Glass-3D^{+BMP} time-course bulk RNA-seq dataset, showing the human CS7 embryo UMAP plot (inset) with colored cells that are transcriptomically similar to each timepoints as indicated (orange – pluripotent cysts/monolayer and 0-, 0.5-, 1-, 3- and 6-hr post-BMP4; green – 12-hr post-BMP4; blue – 24-hr; magenta – 48-hr). While pluripotent and earlier timepoints (orange colored cells) overlaps with Epiblast cells, some 12-hr (green) and all 24-hr (blue) samples are similar to a subpopulation of Primitive Streak cells, and 48-hr (magenta) samples overlap exclusively with Ectoderm (see Materials and Methods for detailed bioinformatics pipeline). (D) *TBXT* expression transposed onto the CS7 human embryo UMAP plot, revealing *TBXT*^{low} sub-population that show transcriptomic similarities to 24-hr post-BMP samples (indicated by dotted circle). (E) An integrated UMAP plot for two *in vitro* cultured gestation date (GD) 14 (8 days after culturing 6-day old blastocyst) cynomolgus macaque peri-gastrula (replotted and reclustered from Yang *et al.*). (F) Transcriptome similarity analysis comparing the GD14 cynomolgus macaque peri-gastrula dataset and the Glass-3D^{+BMP} time-course dataset, showing the Yang *et al.* GD14 UMAP plot (inset “i”) with colored cells that are transcriptomically similar to each timepoints as indicated (orange – pluripotent cysts/monolayer and 0-, 0.5-, 1-, 3-, 6- and 12-hr post-BMP4; blue – 24-hr; magenta – 48-hr). While pluripotent and early datapoints (orange colored cells) overlap with epiblast cells, the 24-hr samples share similarities with *TBXT*^{low} population, and the 48-hr samples overlap with *ISL1*^{high} amnion cells ((G), expression analysis of indicated markers). Gradient scales indicate expression level (orange = high, gray = low). Uncropped plots are found in Fig. S2.

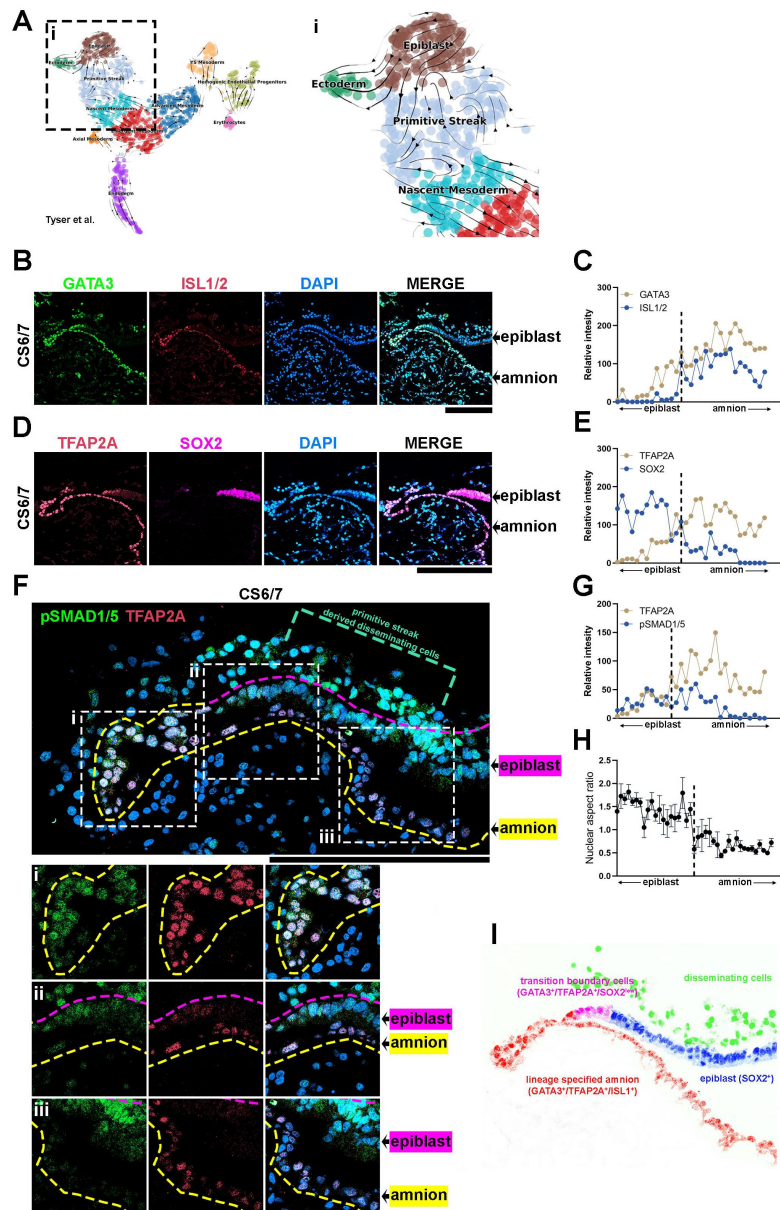


Figure 5.

Transcriptional characterizations of amnion-epiblast squamocolumnar boundary of the CS6/7 cynomolgus monkey amniotic sac.

(A) RNA velocity analysis (scvelo) of the Tyser *et al.* CS7 human embryo dataset, showing predicted lineage progression trajectories from Epiblast to Ectoderm traversing the *TBX1*^{low} population. (B–G) Optical sections of a CS6/7 cynomolgus macaque embryo stained for indicated transcription factors (GATA3, ISL1/2, pSMAD1/5 and SOX2 (B,D,F)), as well as quantitation for relative fluorescent intensity in proximal (closer to epiblast) to distal (closer to amnion) cells of indicated markers at the amnion-epiblast boundary (C, E and G are quantitation of images in B, D and F, respectively). Each dot represents a single cell. (i), (ii) and (iii) indicate insets in (F). Half of the amniotic sac shown. Quantitation shows that, while GATA3⁺/TFAP2A⁺ cells are also SOX2⁺, ISL1⁺ cells are only seen after SOX2 expression is no longer detected: ISL1 expression is exclusive to squamous amnion cells. Moreover, the expression of pSMAD1/5 and TFAP2A is reduced in most distal amnion (ii, iii). Scale bars 200µm. (H) Nuclear aspect ratio quantitation of amnion-epiblast boundary cells in (B), (C) and (D), mean+SEM shown. Dotted vertical line in each plot indicates amnion-epiblast boundary (C,E,G,H). (I) A schematic representation of the CS6/7 cynomolgus macaque amniotic sac: the confocal micrographs in (B) were edited to represent SOX2⁺ epiblast (pseudocolored in blue), primitive streak-derived disseminating (green), GATA3⁺/TFAP2A⁺/SOX2^{low} transition boundary (magenta) and GATA3⁺/TFAP2A⁺/ISL1⁺ lineage specified amnion (red) cells.

While SOX2 expression shows a reducing trend, the expression of GATA3, TFAP2A and ISL1 gradually increases in more distal squamous cells (**Fig. 5B-E**, quantitation in **5C,E**). Furthermore, GATA3, TFAP2A and ISL1 each display distinct expression patterns within the boundary cell population, as the expression of GATA3 and TFAP2A is first detected in columnar SOX2⁺ cells, but in these cells, ISL1 is not observed (**Fig. 5C**). However, a weak but clear expression of ISL1 can be found within ~10 distally located cells, from the first detected GATA3⁺/TFAP2A⁺ cell. Similar observations are seen in another early cynomolgus macaque embryo (**Fig. S3**, staged at CS7). This cell shape change associated with the ordered transcriptional events (from GATA3, TFAP2A then ISL1) is similar to the ordered morphological and molecular changes seen in developing Glass-3D^{+BMP} hPSC-amnion. Thus, the results based on these bioinformatic and expression analyses provide evidence for the presence of amniotic fate progression in early post-gastrula embryos at the boundary between amnion and epiblast (a schematic model shown in **Fig. 5I**).

Gene expression dynamics in

developing Glass-3D^{+BMP} hPSC-amnion

To identify key players potentially involved in amnion fate specification at different stages, we comprehensively characterized all significantly upregulated genes based on when significant upregulations are seen: “immediate” (**Table 2A**, up in 0.5- or 1-hr samples), “early” (**Table 2B**, 3- or 6-hr), “intermediate-1” (**Table 2C**, 12-hr), “intermediate-2” (**Table 2D**, 24-hr) and “late” (**Table 2E**, 48-hr); genes that were kept after a more stringent filtering (described in Materials and Methods) are shown in **Fig. 6**, enabling the identification of additional temporal markers.

Previous studies have identified several *pan*-amnion markers that are known immediate BMP targets (e.g., GATA3 and others in the list of “immediate” genes, **Table 2A**), which are expressed in other early BMP-driven tissue morphogenesis. Among the known *pan*-amnion markers, ISL1 is a highly promising marker for early lineage specified amnion cells (Yang et al., 2021; Zhao et al., 2021; Zheng et al., 2022). In accord with this, we found that ISL1 expression is limited to squamous amnion cells and is not seen in the epiblast or in transitioning boundary cells (**Fig. 5B**). Notably, in Glass-3D^{+BMP}, ISL1 first shows a significant upregulation at 24-hr (intermediate -2, **Fig. 7A**).

This correlates with the timeframe at which squamous cells are first observed (**Fig. 2**). This finding suggests that amnion specification is likely initiated prior to 24 hours in Glass-3D^{+BMP} and that genes that are upregulated between 12-24-hr could be used as additional markers for amnion specification. Consistent with the studies by Tyser et al., we identified DLX5 (716.76-fold increase between 12- and 24-hr, and 2.4-fold increase from 24- to 48-hr, **Fig. 7B**). Interestingly, however, our expression analysis of the Tyser et al. dataset reveals a broader DLX5 expression pattern compared to ISL1 (the DLX5 expression domain map is similar to that of TFAP2A, **Fig. 4B**). Indeed, our IF analyses for DLX5 in developing hPSC-amnion as well as in a CS7 cynomolgus macaque embryo show expression patterns similar to TFAP2A (**Fig. 7C,D**). These results further validate ISL1 as a marker for specified amnion cells and establish that DLX5 as an additional marker for cells undergoing amniogenesis as well as mature amnion cells.

Functional tests of an immediate-, GATA3, and an early-, TFAP2A, responding transcription factors

Immediate- and early-responding transcription factors are functionally relevant candidates that initiate amnion specification and drive the expression of early amnion lineage specified markers such as ISL1. Among the genes in the immediate- and early-responding categories, we screened for genes associated with transcription, and identified several genes, including immediate – GATA3 and early – TFAP2A. As noted above, GATA3 expression precedes TFAP2A expression by approximately 9 hours.

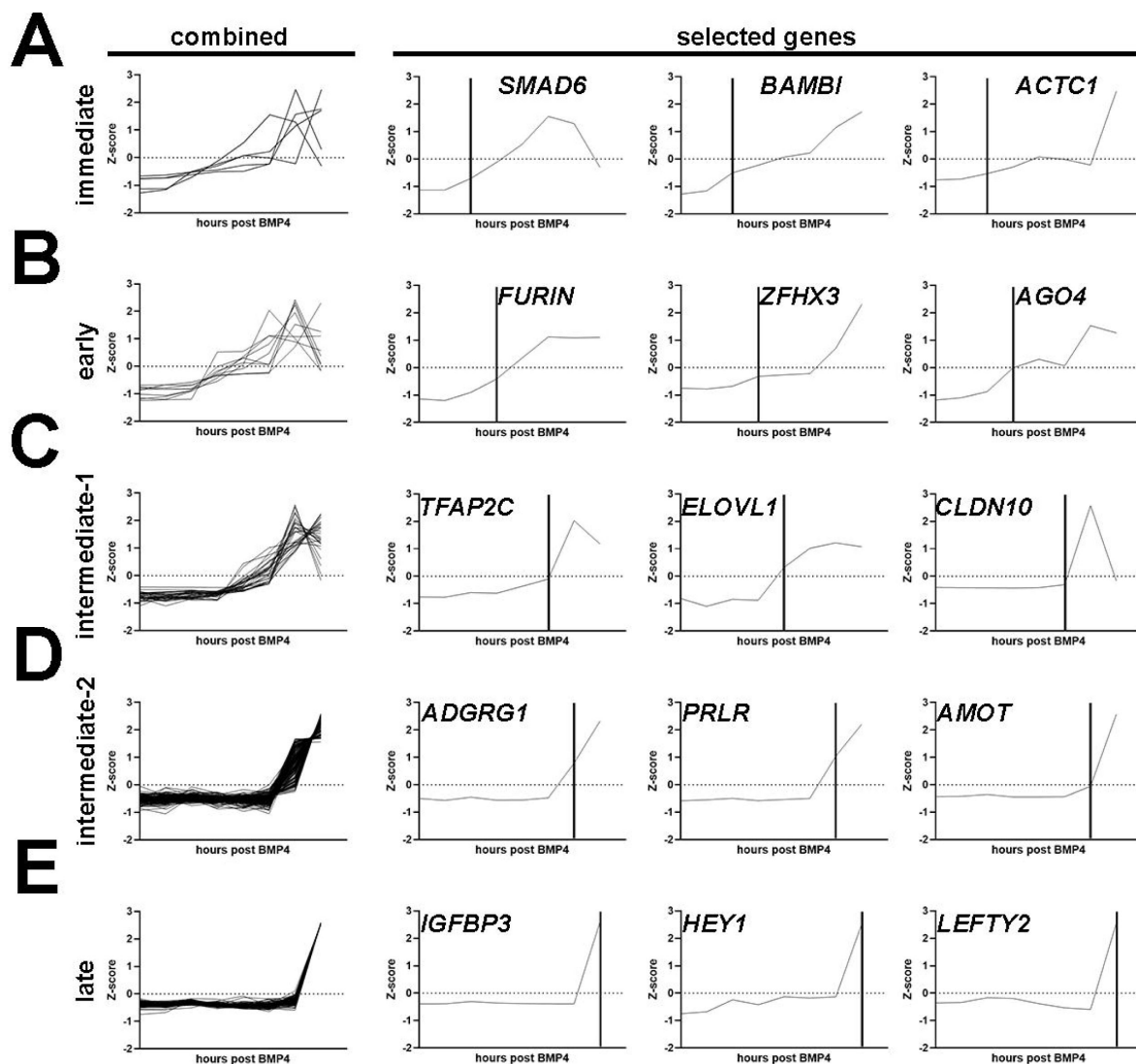


Figure 6.

Characterization of differentially expressed genes identified through the Glass-3D^{+BMP} hPSC-amnion time-course sequencing analysis.

(A-E) Combined and selected genes are shown for each category (A – immediate; B – early; C – intermediate-1; D – intermediate-2; E – late). Genes for each category are selected based on significant upregulation (FDR<0.05, fold>2, based on Z-scores) by 1- (immediate, n = 5), 3- (early, n = 9), 6- to 12- (intermediate-1, n = 30), 24- (intermediate-2, n = 178) and 48-hr (late, n = 157) post-BMP4 treatment, compared to the previous timepoint. Vertical lines indicate timepoints at which significant changes are seen.

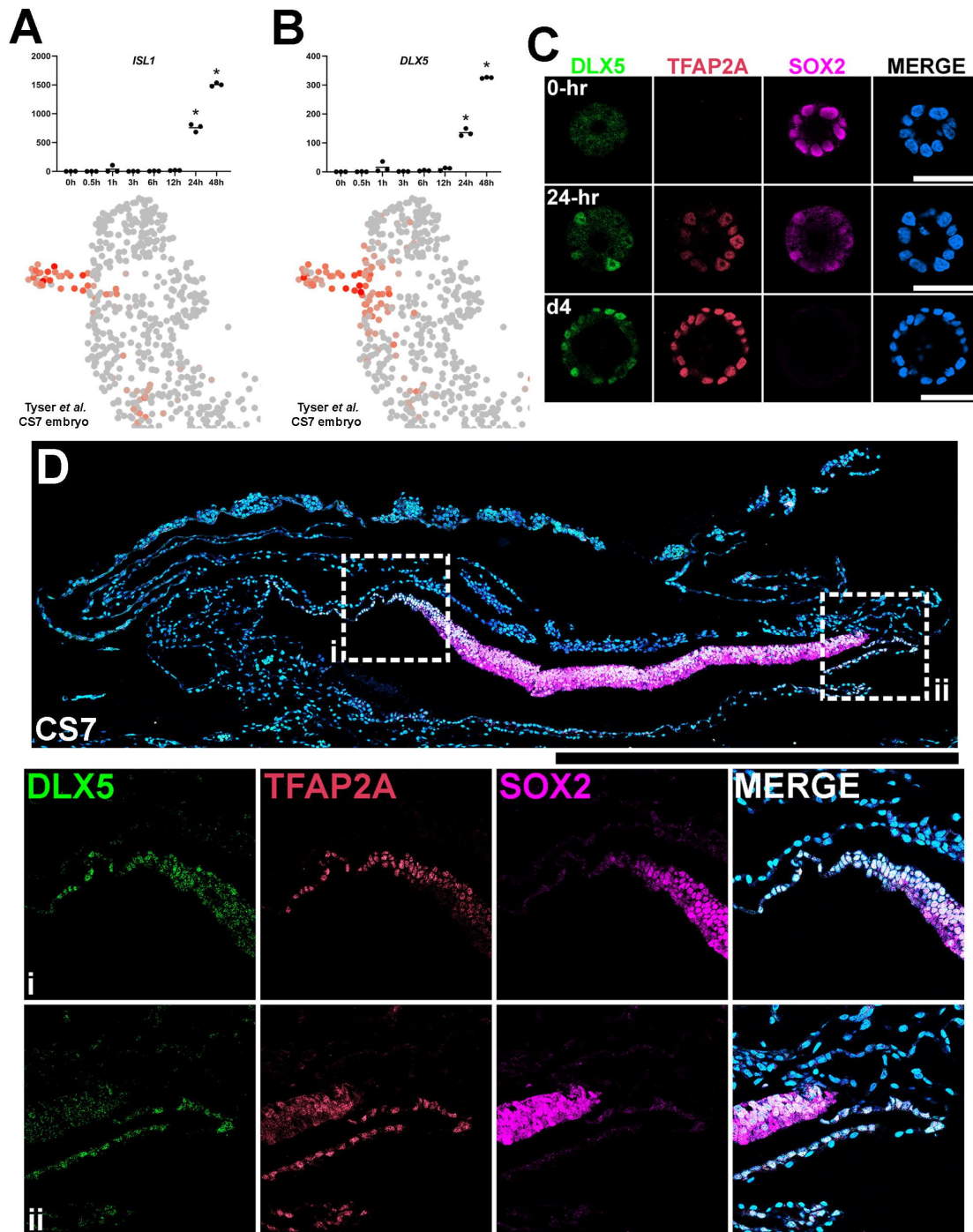


Figure 7.

Establishment of *DLX5* as a new marker of amniogenesis similar to *TFAP2A*.

(**A,B**) Expression analyses for *ISL1* (**A**) and *DLX5* (**B**) in the time-course bulk RNA sequencing (top) and in the single cell CS7 human embryo (bottom) datasets. (**C**) Confocal optical sections of Glass-3D^{+BMP4} hPSC-amnion cysts (at 0-hr, 24-hr (d3) and d4) stained with indicated markers. (**D**) Histological section of a cynomolgus monkey amniotic sac at CS7 stained with indicated markers (*SOX2*⁺ epiblast; *TFAP2A*⁺/*DLX5*⁺/*SOX2*⁻ amnion). Insets (**i** and **ii**) indicate the amnion-epiblast boundaries. *DLX5* and *TFAP2A* share a similar expression pattern at the boundary. Similar to *pSMAD1/5* and *TFAP2A* (shown in Fig. 5F), most distal amnion cells show reduced *DLX5* expression. Surface ectoderm bridging neural ectoderm and amniotic ectoderm is not seen at this stage. Amniotic mesenchyme is observed underlying the *TFAP2A*⁺/*DLX5*⁺ amniotic epithelium at this stage. Scale bars = 500μm.

Both of these factors show highly enriched expression, and both have previously established functions as pioneer transcription factors (Rothstein and Simoes-Costa, 2020 [DOI](#); Takaku et al., 2016 [DOI](#); Tanaka et al., 2020 [DOI](#); Van Otterloo et al., 2022 [DOI](#); Zaret and Carroll, 2011 [DOI](#)). Therefore, we examined the role of GATA3 and TFAP2A by generating hPSC-amnion in Glass-3D^{+BMP} derived from H9 cells lacking GATA3 or TFAP2A (two clonal lines with unique mutations were used for each analysis, see **Fig. S4** for KO validation). Interestingly, *GATA3*-KO hPSC grown in Glass-3D^{+BMP} develop into fully squamous cysts that express amnion markers, perhaps suggesting functional redundancy. In contrast, while controls show uniformly squamous amniotic cells at 48-hr, *TFAP2A*-KO cysts contain both squamous (ISL1⁺) and columnar (NANOG⁺/SOX2⁺) cell populations (**Fig. 8A** [DOI](#) (Glass-3D hPSC-pluripotent cysts), **8B,C** (Glass-3D^{+BMP} hPSC-amnion cysts)). Indeed, a doxycycline inducible expression of TFAP2A in the KO background leads to control-like squamous morphogenesis as well as uniform expression of GATA3 and ISL1 by 48-hr (d4, **Fig. 8C** [DOI](#)). These results suggest that amniogenesis may be halted in the absence of *TFAP2A*. To examine this further at transcriptomic levels, we performed time-course bulk RNA-seq for control and *TFAP2A*-KO hPSC-amnion at 0-, 12-, 24- and 48-hr, and performed a transcriptome similarity analysis using the Tyser *et al.* human CS7 embryo dataset. Indeed, while an expected Epiblast to Ectoderm differentiation trajectory similar to **Fig. 4C** [DOI](#) is found in controls (**Fig. 8D** [DOI](#), left), *TFAP2A*-KO samples do not overlap with Ectoderm cells, even at 48-hr (**Fig. 8D** [DOI](#), right). Rather, 48-hr *TFAP2A*-KO samples show transcriptomic characteristics similar to some Primitive Streak cells (**Fig. 8D** [DOI](#), uncropped plots shown in **Fig. S5**), and, strikingly, our IF analysis shows that some cells robustly expressing TBXT⁺ are seen in *TFAP2A*-KO cysts at 48-hr (**Fig. 8E** [DOI](#)). These results reveal a paused amniogenesis phenotype in the absence of TFAP2A, providing a previously unrecognized notion that TFAP2A is critical for maintaining amnion fate progression.

Discussion

In this study, we developed a reproducible and faithful model for robust mechanistic examinations of human amnion fate specification, and identified a BMP4-dependent amniogenic transcriptional cascade, as well as a role for TFAP2A in maintaining amnion fate progression. Moreover, we established DLX5 as an additional marker for lineage progressing amnion cells and provided evidence for an active amniogenic transcriptional cascade at the epiblast/amnion boundary in peri-gastrula human and non-human primate embryos. These findings and the temporally resolved amniogenic gene lists provided in this study offer additional avenues for further exploration of human amniogenesis.

Previous studies established several broad amnion markers (e.g., GATA3, TFAP2A and ISL1) that are expressed in developing, as well as, differentiated amnion cells. However, comprehensive studies have not been performed to fully characterize how each of these markers corresponds to amnion fate specification steps, especially since BMP signaling is a primary trigger of amniogenesis and a large set of BMP target genes are activated. In this study, a time-course transcriptomic analysis was employed to identify genes that display temporal-specific dynamics among genes that show more static expression patterns: this enabled us to define “stage specific” genes that can be used to distinguish amniogenic fate progression states. In particular, we found that *GATA3* is an immediate BMP target during amniogenesis (along with other known targets such as *ID1*, *ID2* and *BAMBI*); a similar BMP4 → GATA3 transcriptional pathway has been well-established previously in other systems (Abe et al., 2021 [DOI](#); Bonilla-Claudio et al., 2012 [DOI](#); Gunne-Braden et al., 2020 [DOI](#); Vincentz et al., 2016 [DOI](#)).

Early activation of GATA3 is followed by the expression of second-tier “early” genes such as *TFAP2A* by 6-hr (clear TFAP2A protein is detected by 12-hr). Our functional analyses indicate that loss of TFAP2A results in incomplete amniogenesis, with failure to downregulate pluripotent genes, loss of some differentiated amnion markers, and, in some cells, increased expression of TBXT, a primitive streak marker. Thus, TFAP2A, a previously recognized pioneer factor (Rothstein

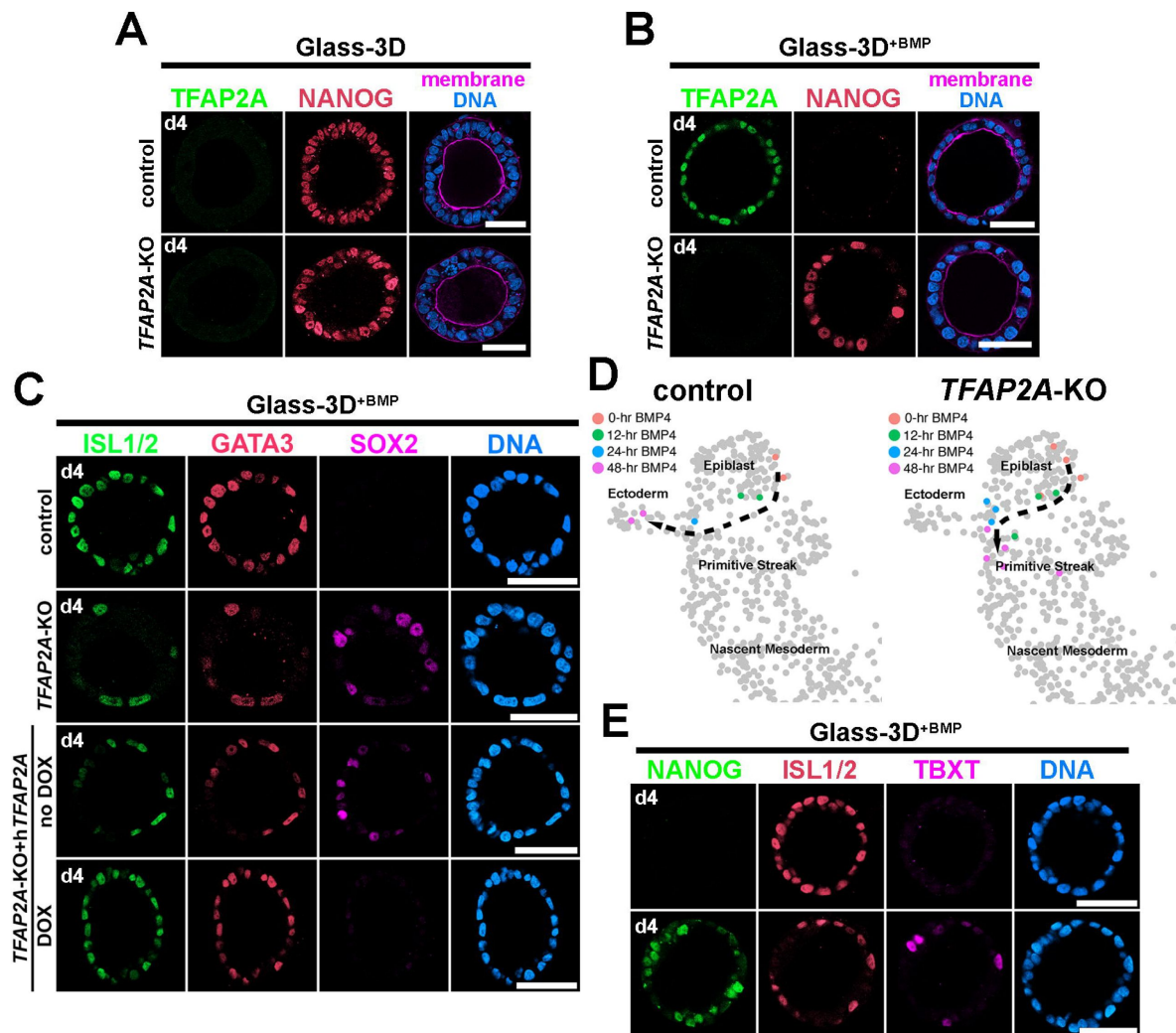


Figure 8.

Analyses of Glass-3D+BMP hPSC-amnion in control and *TFAP2A*-KO background.

(A,B) Optical sections of control and *TFAP2A*-KO cysts in Glass-3D (A, pluripotent) as well as in Glass-3D+BMP (B). While control-like pluripotent cysts are formed in Glass-3D, several NANOG⁺ cells are still seen in Glass-3D+BMP in the absence of *TFAP2A*. (C) Confocal micrographs of d4 control, *TFAP2A*-KO and *TFAP2A*-KO carrying a DOX-inducible human *TFAP2A* transgenic construct with or without DOX treatment, stained with indicated markers. (D) Transcriptome similarity analysis comparing the CS7 human embryo single cell dataset with the time-course (0-, 12-, 24- and 48-hr post-BMP) bulk dataset from control and *TFAP2A*-KO Glass-3D+BMP hPSC-amnion. Orange, green, blue and magenta colored cells are transcriptomically similar to 0-, 12-, 24- and 48-hr post-BMP bulk RNA sequencing samples, respectively. While a clear differentiation trajectory from Epiblast to Ectoderm is seen in control (left), a halted amnion lineage progression is seen in the *TFAP2A*-KO background. (E) Optical sections of control and *TFAP2A*-KO cysts in Glass-3D+BMP stained for NANOG (green), ISL1/2 (red) and TBXT (magenta). Scale bars = 50μm.

and Simoes-Costa, 2020 [\[10\]](#); Van Otterloo et al., 2022 [\[11\]](#)), appears to be an important component that ensures the proper progression of the amniogenesis process, perhaps by suppressing the $TBXT^{high}$ primitive streak-like state.

Interestingly, a recent study by Castillo-Venzor *et al.* reported that, in contrast to our findings, amniogenesis appears intact in the absence of *TFAP2A* (Castillo-Venzor et al., 2023 [\[12\]](#)). However, there are multiple differences between the system utilized by Castillo-Venzor *et al.* vs. the one reported here. Castillo-Venzor *et al.* employ an hPSC-based primordial germ cell-like cell (PGCLC) differentiation culture protocol, in which amniotic and mesodermal cells are also formed. In that system, embryoid bodies are generated from hPSC treated with Activin A and CHIR (a small molecule WNT activator) to form “pre-mesodermal cells”, and then are grown in the presence of exogenous BMP, SCF (stem cell factor, encoded by *KITLG*), EGF (epidermal growth factor) and LIF (leukemia inhibitory factor) as floating aggregates. Moreover, while uniform amniogenesis is seen in Glass-3D^{+BMP} (a characteristic that enables robust mechanistic examinations specifically into amnion fate progression), the PGCLC differentiation culture used by Castillo-Venzor *et al.* leads to the formation of cells of multiple lineages. Therefore, the loss of *TFAP2A* in that system may be compensated by signaling factors provided by mesodermal and/or PGCLC. Alternatively or additionally, it is possible that, in this PGCLC culture, while initial amniogenesis might be present (similar to the focal amniogenesis seen in our system), the later fate spreading event may be absent, perhaps due to differences in tissue geometry (aggregates versus cysts), or due to differences in degrees of lineage commitment by the time that spreading can occur. Together, these results suggest that a single lineage culture system (lacking additional cell types), such as the one we describe here, may more readily expose certain critical events and, therefore, in some cases, may more robustly enable mechanistic investigations into lineage progression.

Several hours following the activation of *TFAP2A*, third-tier “intermediate” genes such as *ISL1* (*ISL1* protein by 24-hr: only in squamous cells) are expressed. It has been previously established that *ISL1* is a key amnion marker in early primate embryos (Yang et al., 2021 [\[13\]](#); Zhao et al., 2021 [\[14\]](#); Zheng et al., 2022 [\[15\]](#)). Interestingly, our IF analyses of early peri-gastrulation cynomolgus macaque embryos show that *ISL1* expression is restricted to amnion cells that are already squamous in nature (Fig. 5 [\[16\]](#)). Additionally, our time-course transcriptomic analyses in Glass-3D^{+BMP} reveal that *ISL1* expression is seen at 12-24-hr, later than *TFAP2A* activation but much earlier than activation of more differentiated amnion markers such as *GABRP*, *EPAS1* and *IGFBP3* (48-hr). Together, these results suggest that amnion cells progress from $GATA3^{+}$, to $GATA3^{+}/TFAP2A^{+}$, to $GATA3^{+}/TFAP2A^{+}/ISL1^{+}$, to mature amnion cells that express these three markers as well as the more mature amnion markers such as *GABRP*, *EPAS1* and *IGFBP3*.

The first amniogenesis event is seen upon implantation, in the epiblast cells underlying the polar trophoblast, establishing the amniotic sac, an asymmetric amnion-epiblast cystic structure (Enders et al., 1983 [\[17\]](#); Enders et al., 1986 [\[18\]](#); Shahbazi and Zernicka-Goetz, 2018 [\[19\]](#); Taniguchi et al., 2017 [\[20\]](#)). In this study, we provide evidence for the active progression of amniogenic fate conversion at the amnion-epiblast boundary of the cynomolgus macaque peri-gastrula. What is the difference between amniogenesis that occurs during implantation and during gastrulation? A previous study by Sasaki *et al.* (Sasaki et al., 2016 [\[21\]](#)) showed that, in a macaque embryo staged at PreG1 (just initiating implantation), a stage before *BMP4* expression is seen in the amnion at PreG2, *BMP2* is abundantly expressed in the visceral endoderm. However, the visceral endoderm underlies the epiblast population that gives rise to the embryo proper (not amnion), and *CER1* is also abundantly expressed in the visceral endoderm (Sasaki et al., 2016 [\[21\]](#)). Thus, *BMP2* in the visceral endoderm in PreG1 embryo is thought not to be the trigger for the initial amniogenesis processes that forms the amniotic sac, and it is currently unclear how BMP signaling is activated in the nascent amnion cells during implantation *in vivo*. Using a Gel-3D hPSC-amnion system, we previously showed that amniogenesis is mechanosensitive, and that a soft substrate can induce BMP signaling in a mechanically dependent manner in developing hPSC aggregates/cysts. This suggests that mechanical signaling might play an important role in the initial BMP signaling

activation and downstream amniogenesis during implantation *in vivo*. In contrast, since a continuous BMP signaling cascade is established in epiblast cells of the early primate gastrula (e.g., driving primitive streak formation, primordial germ cell formation), we speculate that the amniogenesis process that occurs during and post gastrulation may be independent of a mechanical cue. It is, however, important to note that the pluripotency states are distinct in the epiblast cells during implantation compared to during gastrulation; it has been suggested that the epiblast cells are in the formative state during implantation and in the primed state during gastrulation (Kinoshita and Smith, 2018 [↗](#); Shahbazi et al., 2017 [↗](#); Smith, 2017 [↗](#)). Thus, it is possible that the mechanisms regulating responses to BMP ligands, as well as BMP-dependent transcriptional machineries may differ.

Overall, our detailed temporally resolved analyses of Glass-3D^{+BMP} hPSC-amnion development, combined with validation in early macaque embryos has revealed several previously unrecognized findings with implications for BMP-dependent amniogenic transcriptional machineries during amniogenesis, mechanisms continuing amniogenesis in peri-gastrula macaque embryos, and functional characterization of the transcriptional machinery required for amnion fate specification.

Materials and methods

hESC lines

Two human embryonic stem cell lines, H9 (WA09, P30, P48, WiCell; NIH registration number: 0062) and H7 (WA07, P50, WiCell; NIH registration number: 0061), were used in this study. All protocols for the use of hPSC lines were approved by the Human Stem Cell Research Oversight Committee at the Medical College of Wisconsin. All hPSC lines were maintained in a feeder-free system for at least 20 passages and authenticated as karyotypically normal at the indicated passage number. Karyotype analysis was performed at Cell Line Genetics. All hPSC lines tested negative for mycoplasma contamination (LookOut Mycoplasma PCR Detection Kit, Sigma-Aldrich). All transgenic and KO hESC lines in this study used H9 as the parental line. In summary, hESC were maintained in a feeder-free culture system with mTeSR1 medium, or with 50%/50% mix of mTeSR1 and mTeSR plus (STEMCELL Technologies). hESC were cultured on 1% (v/v) Geltrex (Thermo Fisher Scientific), or with Cultrex SCQ (Bio-technie) coated 6 well plates (Nunc). Cells were passaged as small clumps every 4 to 5 days with Dispase (Gibco). All cells were cultured at 37°C with 5% CO₂. Media was changed every day. hESC were visually checked every day to ensure the absence of spontaneously differentiated, mesenchymal-like cells in the culture. Minor differentiated cells were scratched off the plate under a dissecting scope once identified. The quality of all hESC lines was periodically examined by immunostaining for pluripotency markers and successful differentiation to three germ layer cells.

Cynomolgus macaque

Animals

The female and male cynomolgus macaque were housed and cared for at the Wisconsin National Primate Research Center (WNPRC). All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under approval of the University of Wisconsin College of Letters and Sciences and Vice Chancellor Office for Research and Graduate Education Institutional Animal Care and Use Committee (protocol g005061).

Animal breeding and pregnancy detection

Beginning on day 8 post-onset of menses, the female was housed with a compatible male and monitored for breeding. Animals were pair-housed until day 16–20 post-onset of menses. A 2–3 mL blood draw was performed daily from day 8 post-onset of menses until day 16 to assess the timing of ovulation based on the estradiol peak and rise in progesterone in serum. Serum samples were analyzed for estradiol (25 μ L) and progesterone (20 μ L) using a cobas e411 analyzer equipped with ElectroChemiLuminescence technology (Roche, Basal, Switzerland) according to manufacturer instructions. Results were determined via a calibration curve which was instrument-generated by 2-point calibration using traceable standards and a master curve provided via the reagent barcode. Inter-assay coefficient of variation (CV) was determined by assaying aliquots of a pool of rhesus plasma. For estradiol, the limit of quantitation (LOQ) was 25 pg/mL, the intra-assay CV was 2.02%, and the inter-assay CV was 5.05%. For progesterone, the LOQ was 0.2 ng/mL, the intra-assay CV was 1.37%, and the inter-assay CV was 4.63%. A transabdominal ultrasound was performed to detect pregnancy as early as 14 days post-ovulation. The ultrasound measurements in combination with the timing of ovulation were used to estimate the day of conception and gestational age of the pregnancy.

Terminal perfusion uterine collection, paraffin embedding, sectioning and staining

The pregnant female was sedated with intramuscular ketamine (>15 mg/kg) followed by IV sodium pentobarbital (>35 mg/kg) and then perfused with 4% paraformaldehyde (PFA) via the left ventricle. The entire uterus and cervix were removed. The serosa and superficial myometrium were scored for better fixative penetration and to denote dorsal and ventral orientation. Tissues were fixed in 4% PFA with constant stirring and solution changes were performed every 24 hours for a total of 72 h. The uterus was serially sectioned from the fundus to the internal cervical os into 4 mm slices using a dissection box. Cassettes with tissue sections were transferred into 70% ethanol, routinely processed in an automated tissue processor and embedded in paraffin for histological analyses (5 μ m sections). Fixed uterine tissue were cut in 5 μ m thick cross-sections, mounted on slides, deparaffinized in xylene and rehydrated in an ethanol series. Antigen retrieval was performed by boiling in citrate buffer. Sections were blocked 4% goat serum in PBS at RT for at least 3-hr. Subsequent immunolocalization was performed using commercially available primary antibodies, incubated overnight at 4 °C in 4% serum, at the dilutions as shown in **Table 3**. Immunofluorescent detection was performed using secondary antibodies tagged with a fluorescent dye (fluorophores excitation = 488, 555, and 647nm), and counterstained with DAPI. Negative controls were performed in which the primary antibody was substituted with the same concentration of normal IgG of the appropriate isotype. Images were obtained with a Zeiss LSM980 microscope.

Glass-3D^{+BMP} hPSC-amnion formation assays

Glass-3D protocol used to generate hPSC-pluripotent cysts was previously described (Hamed et al., 2023 [\[4\]](#); Shao et al., 2017a [\[4\]](#); Taniguchi et al., 2017 [\[4\]](#); Taniguchi et al., 2015 [\[4\]](#)). In brief, hPSC monolayers were dissociated using Accutase (Sigma-Aldrich) at 37 °C for 10 min, centrifuged and resuspended in mTeSR1/mTeSR plus 50:50 mix containing 10 μ M Y-27632 (STEMCELL Technologies). hPSC were plated as single cells at 20,000 cells cm^{-2} , unless otherwise specified, onto a 1% (v/v) gel coated wells (Geltrex (Thermo Fisher Scientific), or with Cultrex SCQ (Bio-techne)). After 24 hours (on day 1), culture medium was replenished with fresh mTeSR1/mTeSR plus 50:50 media without Y-27632, supplemented with 2% (v/v) Geltrex or with Cultrex SCQ. Thereafter, media change was performed daily until day 4 (using mTeSR1/mTeSR plus 50:50 media supplemented with 1% (v/v) Geltrex or with Cultrex SCQ), unless otherwise noted. This Glass-3D culture condition results in the formation of hPSC-cysts, cysts with a central lumen surrounded by pluripotent columnar cells. The Glass-3D^{+BMP} culture condition was used to generate hPSC-

amnion, cysts with a central lumen surrounded by squamous amnion-like cells. To induce amniogenesis, exogenous BMP ligand (20ng/mL, Bio-technique) was added to developing pluripotent cysts between day 2 to 3 for 24 hours, or between day 2 to 4 for 48 hours, unless otherwise noted (e.g., for time-course analyses). Samples were harvested at day 4 (48-hr since starting BMP4 treatment). LDN-193189 (500nM, STEMCELL Technologies) was used to disrupt BMP signaling.

DNA constructs

piggyBac-CRISPR/Cas9 (pBACON) constructs

A piggyBac-CRISPR/Cas9 (pBACON) vector that contains SpCas9-T2A-puro and hU6-gRNA expression cassettes flanked by piggyBac transposon terminal repeat elements (pBACON-puro), which allows subcloning of annealed oligos containing gRNA sequence at *BbsI* site, has been previously described (Shao et al., 2017b; Townshend et al., 2020; Wang et al., 2021). gRNA targeting genomic sites and oligo sequences to generate pBACON-GFP-hGATA3 (primers: CRISPR_hGATA3_CDS1#1_s and CRISPR_hGATA3_CDS1#1_as) and -hTFAP2A (CRISPR_hTFAP2A_CDS2_s and CRISPR_hTFAP2A_CDS1_as) are found in **Fig. S4** and **Table 3B**; these were designed using publicly available tools (<http://crispr.mit.edu> or https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM).

piggyBac-based transgenic and genome edited hESC lines

PB constructs (3μg) and pCAG-ePBase (Lacoste et al., 2009) (1μg) were co-transfected into H9 hESC (70,000 cells cm⁻²) using GeneJammer transfection reagent (Agilent Technologies). To enrich for successfully transfected cells, drug selection (puromycin, 2μg/mL for 4 days; neomycin, 250μg/mL for 5 days) was performed 48 to 72 hours after transfection. hESC stably expressing each construct maintained the expression of pluripotency markers. For inducible constructs, doxycycline (DOX, 500ng/mL) treatment was performed for 48 hours for neomycin-selected TFAP2A-KO rescue pools using PB-TA-ERN-hTFAP2A(CR)-HA. The TFAP2A gRNA targeted site of human TFAP2A construct (gift of Robert Tjian, Addgene #12100, (Williams and Tjian, 1991)) was mutated using Q5 site directed mutagenesis kit (NEB, primers: SP (RSV) AP2 (hTFAP2A)-CRISPR RES-fw and SP (RSV) AP2 (hTFAP2A)-CRISPR RES-rv). This CRISPR resistant human TFAP2A construct was then PCR amplified (using primers: Clo-dTOPO-hTFAP2A-CRISPR res-fw v2 and Clo-dTOPO-hTFAP2A-CRISPR res-rv v2, HA tagging at C-terminus), which was then cloned into pENTR-dTOPO (Thermo) for Gateway cloning into PB-TA-ERN (Knut Woltjen, Addgene #80474, (Kim et al., 2016)) to generate PB-TA-ERN-hTFAP2A(CR)-HA. Additional plasmid and primer information is found in **Table 3**.

During pBACON-based genome editing, puro-selected cells were cultured at low density (300 cells cm⁻²) for clonal selection. Established colonies were manually picked and expanded for screening indel mutations using PCR amplification of a region spanning the targeted gRNA region (genomic DNA isolated using DirectPCR Lysis Reagent (Tail) (VIAGEN), primers: hGATA3, PCR_GATA3_RI_fw#2 and PCR_GATA3_NI_rv#2; hTFAP2A, PCR_TFAP2A_RI_fw and PCR_TFAP2A_NI_rv), which were subcloned into pPBACAG-GFP (Chen and LoTurco, 2012) at EcoRI and NotI sites, and sequenced (Seq-3' TR-pPB-Fw). At least 12 to 15 bacterial colonies were sequenced to confirm genotypic clonality. Western blots for each protein were also performed to further validate the KO lines. Control cells are H9 hESC in all loss-of-function experiments.

Western blot

SDS PAGE gels (10%) and PVDF membranes were used. Membranes were blocked using Intercept (TBS) Blocking Buffer (Licor), total protein quantification was performed by using Revert 700 Total Protein Stain (Licor), and primary antibody overnight incubation was performed at 4°C, followed

by 30-min IRDye (Licor) secondary antibody incubation at room temperature. Blots were imaged using Licor Odyssey Infared Imaging system.

Immunostaining

hPSC staining protocols have been previously described (e.g., (Hamed et al., 2023 [DOI](#); Shao et al., 2017a [DOI](#); Wang et al., 2021 [DOI](#))). In short, samples (hPSC-cysts, hPSC-amnion) grown on the coverslip were rinsed with phosphate-buffered saline (PBS; Gibco) twice, fixed with 4% paraformaldehyde (Sigma-Aldrich) in 1x PBS for 40 to 60 min, then rinsed with PBS three times, and permeabilized with 0.1% SDS (Sigma-Aldrich) solution for 40 min. The samples were blocked in 4% heat-inactivated goat serum (Gibco) or 4% normal donkey serum (Gibco) in PBS 1 hour to overnight at 4°C. The samples were incubated with primary antibody solution prepared in blocking solution at 4°C overnight, washed three times with PBS (10 min each), and incubated in blocking solution with goat or donkey raised Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific) at room temperature for 2 hours. DNA counterstaining was performed using Hoechst 33342 (nucleus, Thermo Fisher Scientific), Alexa Fluor-conjugated WGA (membrane, Thermo Fisher Scientific). All samples were mounted on slides using 90% glycerol (in 1x PBS). When mounting cyst samples, modeling clays were used as spacers between coverslips and slides to preserve luminal cyst morphology.

Confocal microscopy, live imaging and image analysis

Confocal Images of fixed samples were acquired using Zeiss LSM980 microscope. Measurements of cell nucleus dimensions, cyst epithelial thickness, and cyst orientation angle were performed manually using the Measurement tool in ImageJ (NIH).

RNA isolation and qRT-PCR analysis

Total RNA extraction was performed using TRIzol (Thermo Fisher). The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively. The cDNA was synthesized from total RNA (1µg) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time RT-PCR (qPCR) analysis of mRNA expression was performed using a CFX96 Real-Time PCR Detection System (BioRad) with Power Up™ SYBR Green Master Mix (Applied Biosystems). Primers (**Table 3B**) were designed using NCBI primer design tool to span at least one exon/intron junction. Data were normalized against *GAPDH* and are shown as the average fold increase ± standard error of the mean (SEM) with relative expression being calculated using the $2^{-\Delta\Delta CT}$ method as described previously (Livak and Schmittgen, 2001 [DOI](#)). After amplification, the specificity of the PCR was determined by both melt-curve analysis and gel electrophoresis to verify that only a single product of the correct size was present.

RNA sequencing and bioinformatics

The quality of total RNA was determined using TapeStation RNA ScreenTape. RNA was enriched for mRNA using NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490). cDNA and library preparation were performed using NEBNext® Ultra™ II Directional RNA Library Prep kit for Illumina (New England Biolabs). Library concentration was determined using NEBNext® Library Quant Kit for Illumina (New England Biolabs) or by Qubit (Thermo). Samples were then pooled and were sequenced using Illumina sequencers: NextSeq550 (Versiti Blood Research Institute) or using NovaSeq 6000 (S4 flowcell, the DNA services laboratory of the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign). Sequence data was analyzed using the RNA-Seq workflow in Partek Flow software (Partek Inc., St. Louis, MO, USA). Raw sequencing reads were mapped to the human genome hg38 using STAR version 2.7.8a with default parameter settings. Aligned reads were then quantified to Ensembl Transcripts release 104 using Partek's method and genes with fewer than 10 reads in all samples were excluded from the subsequent

analyses. Gene counts were then filtered based on gene_biotype for protein_coding genes, followed by excluding features where maximum ≤ 30.0 reads. Gene expression was then normalized using DESeq2 (Median Ratio). Differentially expressed genes were selected using as criteria FDR < 0.05 and absolute fold change > 2 .

GO enrichment analysis

GO analysis were performed on significantly upregulated (1,820) or downregulated (2,092) genes in Glass-3D^{+BMP} amnion-cysts vs Glass-3D pluripotent-cysts (From Fig. 3 [↗](#)).

Mapping and gene expression analyses of Tyser et al. Carnegie Stage 7 human embryo single cell RNA sequencing dataset

The original UMAP coordinates and annotations for all 1,195 cells were used throughout. The processed data were downloaded from <http://www.human-gastrula.net> [↗](#), which was used to generate a UMAP plot (using DimPlot in R package Seurat), as well as to perform expression analyses (FeaturePlot in Seurat).

Integration and gene expression analyses of Yang et al. GD14 cynomolgus macaque embryo single cell RNA sequencing dataset

The processed data were downloaded from GSE148683, and the ensemble genome build Macaca_fascicularis_5.0 release 96 was used to identify human orthogonal gene symbols. Datasets from two distinct GD14 embryos (Yang et al., 2021 [↗](#)) were integrated using 5,000 anchor features and 10 dimensions in the IntegrateData function in Seurat R package (v.4.2.1) package: trophoblast cells were removed from the dataset prior to integration. Six general cell populations (epiblast, transition, mesoderm, amnion, endoderm and extraembryonic mesenchyme) were identified using FindClusters function (resolution as 0.4).

RNA velocity (scvelo) analysis of Tyser et al. Carnegie Stage 7 human embryo single cell RNA sequencing dataset

RNA velocities across all 1,195 single cells were embedded on the original UMAP coordinates shown in Tyser *et al.* (Tyser et al., 2021 [↗](#)). The Tyser et al. Carnegie Stage 7 human embryo raw fastq dataset was obtained from ArrayExpress (accession code: E-MTAB-9388). From the aligned BAM files, a loom file was generated using the function run_smartseq2 mode in software velocity (v0.17) with default parameters to create a count matrix made of spliced and unspliced read counts (with Human genome annotation hg19). scVelo (Python package v.0.2.5 (Bergen et al., 2020 [↗](#))) was used to examine inferred RNA velocity and visualized the velocities on the UMAP embedding by stream plot (with default parameters except smooth was set to 1).

Transcriptomic similarity analysis between bulk RNA sequencing and Tyser et al. Carnegie Stage 7 human embryo or Yang et al. GD14 cynomolgus macaque peri-gastrula single cell RNA sequencing datasets

Transcriptomic similarities between bulk RNA sequencing samples and the Tyser *et al.* dataset were examined and visualized using original UMAP coordinates from Tyser *et al.*. In summary, each bulk RNA-seq sample was first downsampled to 100 pseudo-single cell data using R package projectLSI (Granja et al., 2019 [↗](#)), which was followed by data integration with Tyser *et al.* dataset in UMAP dimension reduction space. A medoid from 100 pseudo-single cell data was used as the representation of each bulk RNA-seq sample, and, for each sample, we identified the most transcriptomically similar cell from Tyser *et al.* dataset, based on Euclidean distance on UMAP space. Identical approaches were used for the Yang *et al.* dataset (Yang et al., 2021 [↗](#)), except that the UMAP coordinates were identified using the Seurat package as described above.

Data availability

The raw data, unfiltered count matrix and processed count matrix will be available to the database of Genotypes and Phenotypes (dbGaP) upon publication.

Statistical analyses

Graphs were generated using Prism 7 (GraphPad Software) or R, and statistical analyses were performed using Prism 7. **Fig. 1D**, 5 independent samples were counted at each timepoint (total of > 50 cells/sample). For analysis we used one-way ANOVA with post-hoc Dunnett's multiple comparisons test. In **Fig. 1K**, 3 independent samples were counted (total of > 75 cells were plotted). For analysis we used two-tail Student-T test. In **Fig. 1J**, 3 independent samples were counted (total of > 50 cells were plotted). For analysis we used one-way ANOVA with post-hoc Tukey's multiple comparison test. In **Fig. 2**, relative mRNA expression was detected in n=3 for each timepoint. For analysis we used one-way ANOVA with post-hoc Tukey's multiple comparison test. *, significant (p-value<0.05); ns, non-significant. All experiments were repeated at least three times.

Table 1. Glass-3D^{+BMP} hPSC-amnion time-course bulk RNA sequencing dataset. (A) normalized and filtered count matrix of the time-course bulk-RNA sequencing. (B-C) Gene expression comparison analysis between 48-hr (d4) Glass-3D^{+BMP} hPSC-amnion and d4 Glass-3D hPSC-cysts: lists of 1,813 up-regulated (B) and 2,088 down-regulated (C) genes. (D-E) Gene Ontology analyses (Biological Processes) for top all significantly up- (D) and down- (E) regulated genes.

Table 2. Lists of immediate, early, intermediate and late genes. An excel file containing temporally resolved gene lists: (A) immediate (0.5- and 1-hr, 57 genes); (B) early (3- and 6-hr, 135 genes); (C) intermediate-1 (12-hr, 81 genes); (D) intermediate-2 (24-hr, 642 genes); (E) late (48-hr, 909 genes). Genes that showed significant upregulation compared to the previous timepoint (e.g., 0.5-hr compared to 0-hr, 3-hr compared to 1-hr) were retained. Duplicated genes found in the list from earlier timepoint lists are removed. For example, *GATA3* is significantly upregulated throughout the time course, but is only included in the immediate category. Genes with averaged normalized counts < 100 were excluded.

Table 3. Lists of reagents used in this study. (A-C) Lists of antibodies (A), primers (B) and plasmids (C).

Movie 1. All optical sections of Glass-3D^{+BMP} hPSC-amnion shown in **Fig. 1B**.

Supplementary figure legends

Figure S1. Glass-3D^{+BMP} hPSC-amnion formation using 1196a human induced pluripotent stem cell and H7 human embryonic stem cell lines. (A-C) Confocal micrographs of 1196a (A,B) and H7 lines (C) grown in the Glass-3D^{+BMP} condition, stained with indicated markers and timepoints. Scale bars = 50µm.

Figure S2. Original UMAP plots and TBXT expression analyses. (A,B) Original UMAP expression plots of the Tyser *et al.* CS7 human embryo single cell RNA sequencing dataset used in this study (*GATA3*, *TFAP2A*, *ISL1*, *TBXT* and *DLX5*, A) as well as time-course transcriptome similarity UMAP plot (B). (C) Confocal micrographs of control Glass-3D^{+BMP} cysts stained for ISL1/2 (green), SOX2 (red) and TBXT (magenta). (D) Time-course plot of normalized expression values for *TBXT*. (E,F) Original time-course transcriptome similarity UMAP plot of the Yang *et al.* GD14 cynomolgus

macaque dataset (E) as well as expression plots (F). (G) Optical sections of a CS6/7 cynomolgus macaque embryo stained for TBXT and SOX2. TBXT expression is enriched in primitive streak as well as in the primitive streak-derived disseminating cells.

Figure S3. Quantitation for relative fluorescent intensity in proximal and distal cells of indicated markers at the amnion-epiblast boundary in a CS7 cynomolgus macaque embryo.

Figure S4. Validation of *GATA3*- and *TFAP2A*-KO hPSC lines. (A-C) Sequenced genotyping results of *GATA3*-KO #1 and #2 lines (A), representative sequence traces for each mutations are shown in (B). Western blot analysis for *GATA3* in control and *GATA3*-KO #1 and #2 lysates (C, γ -ACTIN is used as a loading control, triplicates shown). (D-F) Genotyping results of *TFAP2A*-KO #1 and #2 lines (D), representative sequence traces for each mutation are shown in (E). Western blot analysis for *TFAP2A* in control and *TFAP2A*-KO #1 and #2 lysates (F, γ -ACTIN is used as a loading control, duplicates shown).

Figure S5. Uncropped transcriptional similarity UMAP plots for control and *TFAP2A*-KO shown in Fig. 8D [↗](#).

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Competing interests

The authors declare no competing interest.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and the Supplementary Materials. Further information and requests for reagents may be directed to Kenichiro Taniguchi.

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Editors

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Reviewer #2 (Public Review):

In this study, Sekulovski and colleagues report refinements to an in vitro model of human amnion formation. Working with 3D cultures and BMP4 to induce differentiation, the authors chart the time course of amnion induction in human pluripotent stem cells in their system using immunofluorescence and RNA-seq. They carry out validation through comparison of their data to existing embryo datasets, and through immunostaining of post-implantation marmoset embryos. Functional experiments show that the transcription factor TFAP2C drives the amnion differentiation program once it has been initiated.

There is currently great interest in the development of in vitro models of human embryonic development. While it is known that the amnion plays an important structural supporting role for the embryo, its other functions, such as morphogen production and differentiation potential, are not fully understood. Since a number of aspects of amnion development are specific to primates, models of amniogenesis will be valuable for the study of human development. Advantages of this model include its efficiency and the purity of the cell populations produced, a significant degree of synchrony in the differentiation process, benchmarking with single-cell data and immunocytochemistry from primate embryos, and identification of key markers of specific phases of differentiation. Weaknesses are the absence of other embryonic tissues in the model, and overinterpretation of certain findings, in particular relating bulk RNA-seq results to scRNA-seq data from published analyses of primate embryos and results from limited (though high quality) embryo immunostainings.

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Reviewer #3 (Public Review):

In this work, the authors tried to profile time-dependent changes in gene and protein expression during BMP-induced amnion differentiation from hPSCs. The authors depicted a GATA3 - TFAP2A - ISL1/HAND1 order of amniotic gene activation, which provides a more detailed temporary trajectory of amnion differentiation compared to previous works. As a primary goal of this study, the above temporal gene/protein activation order is amply supported by experimental data. However, the mechanistic insights on amniotic fate decision, as well as the transcriptomic analysis comparing amnion-like cells from this work and other works remain limited. While this work allows us to see more details of amnion

differentiation and understand how different transcription factors were turned on in a sequence and might be useful for benchmarking the identity of amnion in ex utero cultured human embryos/embryoids, it provides limited insights on how amnion cells might diverge from primitive streak / mesoderm-like cells, despite some transcriptional similarity they shared, during early development.

[Editors' note: In the revised manuscript, the authors have added new results and made textual revisions that address the reviewers' concerns. These changes have significantly enhanced the clarity, quality, and impact of the study.]

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Author response:

The following is the authors' response to the original reviews.

We appreciate the reviewers for their insightful comments, which have helped to improve the manuscript. We provide specific examples and a point-by-point response to all comments, below. Based on the Reviewers' comments, we revised our manuscript, adding considerable amount of new data (found in Fig. 1A,B, 4E-G, 7C,D, 8C,E, S1B,C, S2C-G, S4C, and Video 1). In the main manuscript text, blue fonts indicate added or revised texts. An additional author (Lauren N. Juga) is added for the newly generated data in the revised manuscript.

Reviewer #1:

Sekulovski et al present an interesting and timely manuscript describing the temporal transition from epiblast to amnion. The manuscript builds on their previous work describing this process using stem cell models.

They suggest a multi-step process initiated by BMP induction of GATA3, followed by expression of TFAP2A, followed by ISL1/HAND1 in parallel with loss of pluripotency markers. This transition was reproduced through IF analysis of CS6/7 NHP embryo.

There are significant similarities in the expression of trophectoderm and the amnion. There are also ample manuscripts showing trophoblast induction following BMP stimulation of primed pluripotent stem cells. The authors should ensure that the amnion indeed is only amnion and not trophectoderm (or the amount of contribution to trophectoderm). As an extension, does the amnion character remain after the 48h BMP4 treatment, and is a trophectoderm-like state adopted as suggested by Ohgushi et al 2022?

Thank you for this insightful comment. As pointed out, Ohgushi et al. showed that, in their culture method, amnion is first induced, and extended culturing leads to the formation of trophectoderm-like cells (Ohgushi et al., 2022).

Importantly, we would like to note that our culture system differs substantially from that of Ohgushi et al. in several respects. First our system uses a 3D culture method while Ohgushi et al. employ 2D hPSC monolayers. Second, the two systems are chemically quite distinct. In our Glass-3D+BMP protocol, cells are cultured in mTeSR media (which contains FGF2 and TGFb1) for two days, by which time they generate 3D pluripotent cysts. BMP is then added to the culture medium for 24 hours, followed by another 24 hours without BMP4. In stark contrast, Ohgushi et al. employ A83-01, an Activin/Nodal signaling inhibitor, and PD173074, an FGF signaling inhibitor (a protocol which they call AP). This treatment leads to spontaneous activation of BMP signaling, but it also clearly inhibits Activin/Nodal and FGF signaling pathways, which remain active in our system. As a result of these distinct chemical as well as

geometrical culturing protocols, their system produces amnion and trophectoderm, while our system produces exclusively amnion.

Further analysis of gene expression data provides additional data supporting our contention that our system produces amnion. Though the gene expression profiles of amnion and trophectoderm are quite similar, specific markers of trophectoderm have been identified including GCM1, PSG1, PSG4 and CGB (Blakeley et al., 2015; Meistermann et al., 2021; Ohgushi et al., 2022; Okae et al., 2018; Petropoulos et al., 2016; Yabe et al., 2016). Importantly, while all of these markers are abundantly expressed in the Ohgushi et al. system, bulk RNA sequencing analysis of our Glass-3D+BMP hPSC-amnion cells reveals that none of these markers are detectable. Indeed, SDC1, a marker that Ohgushi et al. claim distinguishes trophoblast from amnion actually decreases (more than 8-fold) as pluripotent cysts transition to amnion in Glass3D+BMP. Finally, Ohgushi et al. report that ISL1, a key marker of specified amnion population, is initially increased in their system, but is reduced to a basal level overtime. In contrast, in Glass3D+BMP hPSC-amnion, ISL1 expression continuously increases with time, and ISL1 protein expression is seen uniformly throughout the amnion cysts. This uniform expression is also seen in CS6/7 cynomolgus macaque amnion. Together, these results support out conclusion that the Glass-3D+BMP system leads to the formation of amniotic cells, and not trophectoderm cells.

The functional data does not support a direct function of GATA3 prior to TFAP2A and the authors suggest compensatory mechanisms from other GATAs. If so, which GATAs are expressed in this system, with and without GATA3 targeting? Would it not be equally likely that the other early genes could be the key drivers of amnion initiation, such as ID2?

We appreciate this helpful comment. We agree that our data do not provide sufficient evidence for the role of GATA3 in early amniogenesis. We also agree that other early genes could be key drivers, and apologize for including our speculation that focuses only on GATA2. GATA2 was selected because, among the other GATAs, GATA2 and GATA3 are the only abundantly expressed GATA factors. This point suggesting a potentially redundant role of GATA2 is now removed from the manuscript (Line#355 of the original manuscript).

The targeting of TFAP2A displays a very interesting phenotype which suggests that amnion and streak share an initial trajectory but where TFAP2A is necessary to adopt amnion fate. It would again be important to ensure that this alternative fate is indeed in streak and not misannotated alternative lineages, including trophoblast.

Is TBXT induced in this setting as well as in the wt situation during amnion induction? This should be displayed as in Figure 3D and would be nice to be complimented by NHP IF analysis.

We will address these two closely related comments together.

TFAP2A-KO cysts contain ISL1+ squamous cells as well as SOX2+ pluripotent cells, suggesting that, while the initial focal amniogenesis is seen, subsequent spreading event is not seen. Interestingly, our new data show that TFAP2A-KO cysts display cells with high TBXT expression (Fig. 8E, Line#373-374). This result suggests that, in the absence of TFAP2A, once amnion lineage progression is halted, more primitive streak-like (TBXThigh) lineage emerges. It is important to note that TBXT expression is not seen in the trophectoderm population of cynomolgus macaque peri-gastrula (Sasaki et al., 2016; Yang et al., 2021).

As suggested, we now include a TBXT expression time course during hPSC-amnion formation in Fig. S2D of the revised manuscript. These data show weak TBXT expression (transcripts) starting at the 24-hr timepoint. However, a clear TBXT protein signal could not be detected using IF (Fig. S2C), likely because TBXT expression is very low (Line#264-265). While

statistically significant compared to the 12-hr timepoint, TBXT expression is 31 FPKM $\pm$ 0.8 (standard deviation) at 24-hr and 48 FPKM $\pm$ 6 at 48-hr. These are low expression values compared to, for example, TFAP2A, which displays 572 FPKM $\pm$ 23 at 12-hr and 1169 FPKM $\pm$ 27 at 24-hr, at which TFAP2A is readily detected using IF. While weak nuclear TFAP2A is seen using IF at 6hr (187 FPKM $\pm$ 7), no clear TFAP2A is detected at 3-hr (74 FPKM $\pm$ 7). Another example is ISL1, which displays 758 FPKM $\pm$ 55 at 24-hr and 1505 FPKM $\pm$ 26 at 48-hr, when ISL can be detected using IF. Importantly, we were not able to detect ISL1 protein expression using IF at

12-hr, at which its expression level is 12 FPKM $\pm$ 18. Lastly, we now show that, in the cynomolgus macaque peri-gastrula, while pSMAD1/5+ primitive streak-derived disseminating cells show abundant TBXT expression, no clear TBXT expression is seen in the amnion territory (Fig. S2G, Line#291-293).

Together, these results show that while a TBXT_{low} state clearly emerges during hPSC-amnion development, in wild-type hPSC cultured in Glass-3D+BMP, TBXT levels remain low throughout amnion differentiation. However, in the absence of TFAP2A, a TBXT_{high} state is seen, suggesting that TFAP2A is critical for suppressing this TBXT_{high} state in fate spreading cells, perhaps by preventing BMP responding cells from acquiring embryonic lineages (e.g., mesodermal and/or primordial germ cells).

The authors should address why they get different results from Castillo-Venzor et al 2023
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Thank you very much for this helpful suggestion, and we now include a section detailing this in the Discussion (Line#410-432). In short, we propose several possibilities. First, culturing conditions are highly distinct. Castillo-Venzor et al. (Castillo-Venzor et al., 2023) utilize initial “pre-mesoderm” conditioning by Activin and CHIR, followed by treating floating embryoid bodies with a growth factor cocktail (BMP, SCF, EGF and LIF). In contrast, our system (Glass-3D+BMP) employs BMP stimulation of pluripotent cysts. Thus, we suspect that, in the PGCLC differentiation condition, cells are conditioned to the pre-mesodermal lineage. Moreover, we propose that amnion fate spreading may not be present in the PGCLC system, perhaps due to differences in geometry (aggregates versus cysts), or due to differing lineage commitment programs. That is, while initial amniogenesis is seen in the PGCLC system, most cells may already be committed to the PGC-like or mesodermal lineages by the time amnion fate spreading can occur. Alternatively, because several cell types (PGC-like, mesodermal and amniotic) co-exist in the culture by Castillo-Venzor et al., PGC-like and/or mesodermal cells may compensate for the loss of TFAP2A.

Reviewer #2:

In this study, Sekulovski and colleagues report refinements to an in vitro model of human amnion formation. Working with 3D cultures and BMP4 to induce differentiation, the authors chart the time course of amnion induction in human pluripotent stem cells in their system using immunofluorescence and RNA-seq. They carry out validation through comparison of their data to existing embryo datasets, and through immunostaining of post-implantation marmoset embryos. Functional experiments show that the transcription factor TFAP2C drives the amnion differentiation program once it has been initiated.

There is currently great interest in the development of in vitro models of human embryonic development. While it is known that the amnion plays an important structural supporting role for the embryo, its other functions, such as morphogen production and differentiation potential, are not fully understood. Since a number of aspects of amnion development are specific to primates, models of amniogenesis will be valuable for the

study of human development. Advantages of this model include its efficiency and the purity of the cell populations produced, a significant degree of synchrony in the differentiation process, benchmarking with single-cell data and immunocytochemistry from primate embryos, and identification of key markers of specific phases of differentiation. Weaknesses are the absence of other embryonic tissues in the model, and overinterpretation of certain findings, in particular relating bulk RNA-seq results to scRNA-seq data from published analyses of primate embryos and results from limited (though high quality) embryo immunostainings.

We are happy that Reviewer #2 agrees that our Glass-3D+BMP model is important for investigating additional roles of amniogenesis, as well as roles of amnion as a signaling hub, due to the purity of the amniotic cell population, and a high degree of synchrony of differentiation.

We respectfully disagree that the absence of other embryonic tissues in the model is a weakness: rather, we believe it is a strength because this single lineage amnion model allows us to directly (and independently) investigate mechanisms underlying amnion lineage progression. For example, as noted above in our response to Reviewer #1, use of our hPSCamnion model allowed us to see a very specific and interesting phenotype in the absence of TFAP2A (reduced amnion formation and emergence of an alternative lineage), though previous findings by Castillo-Venzor et al. concluded that amniogenesis is not affected by loss of TFAP2A. We noted that the culture method used by Castillo-Venzor et al. contains several cell types (amniotic, mesodermal and PGC-like), and that amniogenesis may be intact in that model due to compensation by the presence of these other cell types. That is, while cell-cell interactions can indeed be gleaned in culture systems with several cell types, the presence of multiple cell types and their additional signaling inputs can also confound some aspects of mechanistic investigations. We now include a paragraph in the Discussion of the revised manuscript (Line#410-432), in which we detail these ideas, and suggest that, because of the cell purity, our Glass-3D+BMP model enables robust mechanistic examinations, specifically during amnion formation.

We address Reviewer #2's point about bulk vs. single cell transcriptomic similarity analysis in Reviewer's specific point #4 below. We do, however, want to note here that we have performed the same analysis using a 14-day old cynomolgus macaque peri-gastrula single cell RNA sequencing dataset generated by Yang et al. (Yang et al., 2021), and obtained a lineage trajectory (Fig. 4F, Line#265-268) similar to that seen when the Tyser et al. dataset (Tyser et al., 2021) was used (Fig. 4C).

Importantly, while cynomolgus macaque early embryo samples are limited, we now include additional staining (Fig. S2G).

Reviewer #2 (Recommendations For The Authors):

Provide more confirmation of key findings in more than one stem cell line.

We now confirm key findings in the H7 human embryonic stem cell line (Fig. S1C).

Provide stronger evidence e.g. scRNA-seq to support the existence of intermediate cells or tone down the conclusions.

We agree that this is a very important point. In our recent study (Sekulovski et al., 2023), we performed single cell RNA sequencing of Gel-3D, another hPSC-amnion model. In this study, we comprehensively described the transcriptome associated with the “intermediate” cell types, as well as CLDN10 as a marker of these cell types. Moreover, we now include additional data showing the molecular characteristics of the TBXTlow intermediate cells during

amniogenesis in hPSC-amnion (Fig. S2C, S2D) and d14 cynomolgus macaque peri-gastrula (Fig 4G, replot of single cell RNAseq by (Yang et al., 2021), Line#264-268).

Provide more data on the expression of DLX5 in the model.

We now provide a DLX5 staining time course in Fig. 7C. We find that, similar to ISL1, prominent DLX5 staining is seen in the focal cells at 24-hr post-BMP. Interestingly, at 48-hr, while some cells show high levels of DLX5, some cells show low DLX5 levels; this is of an interest for future investigations.

(1) L159 - the authors should repeat more of the key results in at least one other hPSC line, to ensure reproducibility of the method. Figure S1 contains minimal information (one timepoint, three genes, one biological replicate) on a single different hPSC line.

We now include additional validation analysis using the H7 human ESC line (Fig. S1).

(2) Figure 1- it is a little difficult to appreciate cyst formation from images taken at one level in the stack, can the authors perhaps show a 3D rendering or video to display morphogenesis better?

We now provide all optical sections of cysts shown in Movie 1.

(3) Figure 1-did the authors carry out podocalyxin staining? This is a standard marker for lumenogenesis.

We now provide PODXL staining (Fig. 1A,1B).

(4) L248 onwards and Figure 4-I am a little skeptical concerning conclusions drawn from an overlay of bulk RNA-seq onto scRNA-seq UMAP plots. I think the authors need to provide some strong justification for this approach. I would be particularly careful about concluding that cells depicted in Fig 4D represent an intermediate close to primitive streak and even more careful about claiming any lineage relationship between T-positive "primitive streak like intermediates" and the trajectory of cells in the model. UMAP is a dimension-reduction technique for the visualization of clusters in high-dimensional data. It is not a lineage-tracing methodology. It would have been preferable for the authors to present their own scRNA-seq data from the model.

We are sorry that it was not clear that our approach to find similarity between bulk and single cell RNA-seq data is largely based on a published work (Granja et al., Nature Biotechnology 2019, (Granja et al., 2019)) named projectLSI. Please refer to our Methods section for details of the implementation and how we modified it for better visualization (addressed in Line#667-676 of the original manuscript, now in Line#718-730). The performance of projectLSI was extensively evaluated in the original article. Furthermore, as pointed out, UMAP is indeed a dimension reduction method that has been widely used in single cell RNA-seq research. In addition to visualizing clusters, trajectory analysis, such as RNA-velocity (which is used in this study), is another successful and widely adapted application of UMAP to gauge fate progression. Therefore, we believe that UMAP can be effectively used as a lineage prediction methodology, and that our use of bulk to single cell transcriptomic similarity analysis leveraging projectLSI is well justified at conceptual and technical levels.

As illustrated in Fig. 5A, we performed RNA-velocity analysis of the Tyser et al. dataset, and our result clearly predicts a differentiation trajectory from Epiblast, a part of the TBXTflow population shown in Fig. 4D, and, then, to Ectoderm/Amnion cells. Consistent with this bioinformatic result, we now show that some cells show some but weak TBXT expression (at

the transcript level) at the 24-hr post-BMP timepoint in control hPSC-amnion (Fig. S2D, Line#264-265). Importantly, our conclusion is drawn from a trajectory based on our time course (0, 0.5, 1, 3, 6, 12, 24, and 48 hours post-BMP treatment) which shows a clear transition from epiblast cells to TBXTlow and then finally to the ectoderm/amnion population. Moreover, using the transcriptomic similarity analysis, we found that the loss of TFAP2A leads to emergence of more primitive streak-like transcriptional characteristics (Fig. 8D). Indeed, using IF, we now show that several fate spreading cells in the TFAP2A-KO cysts are TBXThigh (Fig. 8E, Line#373-374). Thus, the new data provide additional evidence for the successful implementation of this bulk/single cell transcriptomic similarity analysis.

Together, our bioinformatic and localization analyses show that the Glass-3D+BMP system recapitulates the trajectory found in our Tyser et al. RNA-velocity analysis, further supporting the validity of this differentiation trajectory. To avoid confusion, however, we now omit the “primitive streak-like” phrase when describing the TBXTlow cells because, while they may show some TBXT expression, they are likely intermediate fate transitioning cells. Indeed, a recent study by Ton et al. (Ton et al., 2023) showed that the Tyser et al. Primitive Streak cells consist of a mix of several lineage progressing cells (e.g., Epiblast, Non-neural ectoderm, Anterior or caudal primitive streak, PGC). Therefore, these cells are now specifically described as “TBXTlow” state; TBXThigh cells are described as primitive streak-like state.

| (5) L276 Tyser data do come from a primate model; the authors mean NHP.

We now specifically state that the validation is performed in a non-human primate model (Line#280).

| (6) Figure 5-though the immunostaining of the CS6/7 monkey embryos is excellent, the authors should not overinterpret these images. What is shown is not a time course, and one can only infer that a particular pattern of gene expression exists in a spatial sense from these images. In the model (Figure 2), the epiblast markers gradually fade and overlap for a time with emergent amnion markers, but in Figure 5 the transition between epiblast and amnion in the embryo seems pretty sharp, at least in terms of gene expression. There may be a few cells in D that show overlap of SOX2 and TFAP2A, but if the authors want to claim that a transition zone exists, they need to produce stronger evidence. Figure 7 is more convincing but see the next point.

Thank you for this insightful comment. We now address the nature of the transitioning boundary cell population extensively in our other recent study (Sekulovski et al., 2023).

| (7) Figure 7 further confuses the issue. A zone at either end of the epiblast is clearly positive for Sox2 and the two amnion markers, clearer than in Figure 5, but why does the marker DLX5 overlap with SOX2 in the embryo (7d) but not the model (7C)? Arguments regarding intermediate cell populations would be greatly strengthened by scRNA-seq data on the model system.

In our original manuscript, our DLX5 staining was performed at 48-hr post-BMP, at which SOX2 expression is absent in all cells. Our new analysis at the 24-hr timepoint now shows that DLX5 is expressed in SOX2+ cells (this is now presented in Fig. 7C).

As stated in the point #6, our recent study comprehensively describes the transcriptomic and spatial characteristics of the transitioning boundary cell population (Sekulovski et al., 2023).

| (8) L357 TFAP2C KO does not resemble intermediate cysts in Figure 2. In Figure 2, both SOX2 and amnion markers are co-expressed in the same cells. In 8C, SOX2 and ISL1 are mutually exclusive.

We agree with this comment, and now removed this statement pointing out the resemblance (Line#359 of the original manuscript).

(9) Figure 8d-the same caveats noted above regarding the interpretation of superposition of bulk RNA-seq data with scRNA-seq UMAP analysis apply here.

Please refer to our explanation in point#4.

Reviewer #3:

In this work, the authors tried to profile time-dependent changes in gene and protein expression during BMP-induced amnion differentiation from hPSCs. The authors depicted a GATA3 - TFAP2A - ISL1/HAND1 order of amniotic gene activation, which provides a more detailed temporary trajectory of amnion differentiation compared to previous works. As a primary goal of this study, the above temporal gene/protein activation order is amply supported by experimental data. However, the mechanistic insights on amniotic fate decision, as well as the transcriptomic analysis comparing amnion-like cells from this work and other works remain limited. While this work allows us to see more details of amnion differentiation and understand how different transcription factors were turned on in a sequence and might be useful for benchmarking the identity of amnion in ex utero cultured human embryos/embryoids, it provides limited insights on how amnion cells might diverge from primitive streak / mesoderm-like cells, despite some transcriptional similarity they shared, during early development.

We are happy that Reviewer #3 appreciates that our model can be used effectively to identify previously unrecognized amniotic gene activation cascade, providing a comprehensive timecourse transcriptomic resource.

As detailed below, we address specific concerns raised by Reviewer #3. We now provide additional mechanistic insights into amnion fate progression, and include additional transcriptomic comparisons with a cynomolgus macaque single cell RNA sequencing dataset.

Reviewer #3 (Recommendations For The Authors):

(1) The authors generated KO cell lines lacking GATA3 and TFAP2A, respectively. Their results showed some disrupted amnion differentiation only in TFAP2A-KO. Therefore, these data do not provide sufficient evidence to support whether these transcription factors are crucial for amnion fate specification. Perhaps an experiment could be done with overexpression of these markers and testing if they could force hPSC to adopt amnion-like fate.

Thank you for this insightful comment. We generated cell lines that enable us to inducibly express GATA3 or TFAP2A, and the transgene expression was induced at d2 (when BMP treatment is normally initiated) until d4. However, this inducible expression did not lead to amniogenesis, and cysts maintained pluripotency. Due to the uninterpretable nature, these results are not included in the revised manuscript.

As detailed extensively in the manuscript, within each cyst, amniogenesis is initially seen focally, then spreads laterally resulting in fully squamous amnion cysts. This is also seen in our previously published Gel-3D amnion model (extensively described in (Shao et al., 2017)). In the absence of TFAP2A, we showed that the focal amniogenesis is observed, but spreading is not seen, suggesting that TFAP2A controls amnion fate progression. Therefore, while TFAP2A is not critical for the amnion fate specification in the focal cells, our results show that TFAP2A indeed helps to promote amniotic specification of cells neighboring the focal

amniotic cells. Moreover, in the revised manuscript, we now show that TFAP2A transgene expression in the TFAP2A-KO background restores formation of fully squamous hPSC-amnion, further establishing the role of TFAP2A in amnion fate progression (Fig. 8C of the revised manuscript, Line#362-364).

(2) The transcriptomic analysis made by the authors provides some comparison between BMP-induced amnion-like cells in vitro and the amnion-like cells from CS7 human embryo in vivo. However, the data set from the human embryo contains only a limited number of cells, and might not provide a sufficient base for decisive assessment of the true identity of amnion-like cells obtained in vitro. It might help if the authors could integrate their bulk sequencing data with other primate embryo data sets.

Thank you for this helpful comment. We have now performed our transcriptional similarity analysis using early (day 14) cynomolgus macaque embryo datasets generated in a study by (Yang et al., 2021), and found that the bulk time-course transcriptome of our hPSC-amnion model overlaps with the cynomolgus macaque amniotic lineage progression (Fig. 4F, Line#265268). We also now provide the expression of key markers within the Yang et al. dataset (GATA3, TFAP2A, ISL1, TBXT, DLX5, Fig. 4G, S2F).

(3) Following the point above, the authors used transcriptomic analysis to identify several intermediate states of cells during amnion differentiation and claimed that there is a primitivestreak-like intermediate. However, this might be an overstatement. During stem cell culture and differentiation, intermediate states showing a mixture of biomarkers are very common and do not imply that such intermediates have any biological meaning. However, stating that amnion differentiation passes through primitive streak-like intermediates, might imply a certain connection between these two lineages, for which there is a lack of solid support. Instead, a more interesting question might be how amnion and primitive streak differentiation, despite some transcriptomic similarity, diverge from each other during early development. What factors make this difference? The authors might further analyze RNA-seq data to provide some insights.

Thank you very much for the insightful comments.

We understand Reviewer #3's concern that the intermediate state that we see may not recapitulate a primitive streak-like state. However, in our original manuscript, we described these cells as "Primitive Streak-like" because those cells were annotated as Primitive Streak in the dataset by Tyser et al. Interestingly, a recent study by Ton et al. showed that the Tyser et al. Primitive Streak cells actually consist of a mixture of different cell lineages (e.g., Epiblast, Nonneural ectoderm, Anterior or caudal primitive streak, PGC (Ton et al., 2023)). Therefore, we agree that it was an overstatement to call them "Primitive Streak-like", and, to avoid confusions, we now label the TBXT^{low} sub-population found in the Tyser et al. Primitive Streak population as "TBXT^{low} state" throughout the manuscript.

Our data indicate that TFAP2A may play a role in controlling the lineage decision between amnion and primitive streak cells that abundantly express TBXT (TBXT^{high}). In the original manuscript, we included data showing that 48-hr TFAP2A-KO cysts show transcriptomic characteristics similar to some Primitive Streak cells (Fig. 8D). Intriguingly, our new data show that, in the absence of TFAP2A, some TBXT^{high} cells are indeed seen (Fig. 8E, Line#373-374). These results provide a body of evidence for the role of TFAP2A in promoting the amniotic lineage, perhaps by suppressing the TBXT^{high} state. This point is now addressed in the Discussion (Line#401-409).

Additional new data:

Using Western blot, we now show that GATA3 is absent in the GATA3-KO lines (Fig. S4C). We noticed that this was lacking in the original manuscript.

We now show that an inducible expression of TFAP2A in the TFAP2A-KO cysts leads to controllike cysts (Fig. 8C, Line#362-364).

Additional changes:

Typos were fixed in Fig. 5I – “boundary” and “disseminating” were not spelled correctly.

Line#350 – we originally noted “GATA3 expression precedes TFAP2A expression by approximately 12 hours”. This was incorrect, and is changed to 9 hours in the revised manuscript. We apologize for this mistake.

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